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13. ABSTRACT (Maximum 200 Words)

Because of the potential synergistic interaction between an anti-angiogenic aminosterol, squalamine, and other angiogenic modifiers such as vascular endothelial growth factor (VEGF) and cytokines that may be released during intermittent androgen withdrawal therapy, we tested extensively the interaction between squalamine and VEGF for an enhanced cytotoxicity to human prostate cancer cells in vitro and xenografts tumor models in vivo. While in vitro synergistic interaction was demonstrated specifically in human prostate cancer cell lines containing a functional androgen receptor, we encountered difficulty in demonstrating such synergism in vivo for the reason that severe toxicity was noted when VEGF was delivered as an Ad-CMV-TK vector. For this reason, we explored the other possible synergistic interaction between squalamine and castration. **Results and Discussion:** Squalamine is highly synergistic to castration-induced endothelial destruction when applied at the time of castration. We noted VEGF receptor, flt-1 and integrin profile (e.g. $\alpha 6\beta 4$) can predict squalamine response. Prostate cancer cells lacking the expression of these markers may be less responsive to the synergistic interaction between squalamine and castration. We are currently assessing the possible interaction between squalamine and VEGF and squalamine and androgen status of the cell culture and in animals subjected to castration to evaluate if synergism may exist particularly against the growth of endothelial cells.

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Introduction:

The objective of this proposal is to seek for a combination therapy between a low molecular weight aminosterol squalamine which has anti-angiogenic activity against induced endothelial proliferation and migration and vascular endothelial growth factor, VEGF, on the growth of human prostate tumors both *in vitro* and *in vivo*. Although both agents when applied alone have little anti-tumor effect, they have remarkable synergistic action when applied together in tumor cells that express certain profiles of integrin isotypes and VEGF receptors. This approach is taken because of the known inherent genetic stability of endothelial cells which are required for tumor cells' continued growth and expansion and the potential clinical application of an effective combination therapy targeted at tumor and its endothelial supplies for the effective treatment of hormone refractory prostate cancers.

Body:

Task 1: Establishment of in vivo human prostate tumors:

This task has been completed. Please see previous progress report.

Task 2: Construction, characterization and production of adenoviruses that contain VEGF driven by a CMV universal promoter:

This task has been completed. Please see previous progress report.

Task 3: Evaluation of the *in vitro* and *in vivo* synergism between squalamine and VEGF (or castration), and assessment of the biochemical and morphologic changes of the prostatic tissues in vivo:

This task has been completed. Please see previous progress report.

Task 4: Determine the *in vitro* effect of squalamine and/or VEGF on the growth of prostatic and endothelial cells:

This task has been completed as indicated in Appendix 1, (Jin et al. *Cancer Gene Therapy* in press, 2005).

Task 5: Recording of the morphologic changes of cells after squalamine and/or VEGF treatment:

This task has been completed. Please see previous progress report.

Task 6: Evaluation of the relationship between morphologic changes of prostate cancer and endothelial cells *in vitro* after squalamine and/or VEGF treatment with that of their biochemical expression of TSP-1 and cell surface integrin isotypes:

In a previous progress report, we have completed the analysis of morphologic and biochemical features of prostate tumors after squalamine treatment in intact and castrated hosts. We are presently evaluating TSP-1 and cell surface integrin expression by prostate cancer cell lines and tumor specimens following treatment with squalamine and/or VEGF anti-androgenic therapy.

Task 7: Confirmation of the above biochemical responses of prostate cancer cells and endothelial cells to squalamine and VEGF *in vivo*.

We have completed the evaluation of HUVEC cell culture *in vitro* to the effect of squalamine. We are presently testing the effect of squalamine and VEGF on chimeric tumor growth *in vivo*.

Task 8: Evaluation of methodologies for evaluating signal cascade and apoptosis following VEGF and squalamine.

This task has been completed. We are in the process of preparing a manuscript indicating the effect of VEGF and squalamine on phosphorylation of PP125FAK and Pyk2.

Task 9: Evaluation of changes in signal transduction components following exposure to squalamine and/or VEGF *in vitro* and confirmation of such changes in prostate tumor models *in vivo*.

We have developed a basic system in evaluating the signal transduction of squalamine and/or VEGF *in vitro* in prostate and endothelial cell culture *in vitro*. The *in vivo* work is currently under study.

Task 10: Characterization of changes of signal transduction components and their relationship to apoptosis, and comparison of their activity both *in vivo* and *in vitro*.

This task is currently under investigation.

Key Research Accomplishments:

- We have completed a manuscript indicating co-targeting tumor and tumor-associated endothelium using gene therapy as a prototype resulted in improved affect in eradicating cancer growth as xenografts in culture. This same study also has been confirmed *in vitro* (Jin et al. *Cancer Gene Therapy*, in press, 2005).
- We are establishing the basic methodology to study signal transduction in prostate cancer cells and the same methodologies will be used to measure signaling pathway in response to squalamine and/or VEGF.

Reportable Outcomes:

1. A manuscript by Jin et al. was accepted for publication in *Cancer Gene Therapy.*
2. A review article by Chung et al. was accepted by *Journal of Urology* Jan. 2005.

Conclusions:

VEGF and squalamine synergism appears to be a phenomenon *in vitro* and its *in vivo* synergism is more difficult to demonstrate due to severe toxicity of delivery of VEGF to tumor tissues in tumor-bearing animals. The concept to enhance tumor and endothelial cell death using angiogenic modifiers however received support by the application of squalamine immediately after castration. Based on immunohistochemical data, it appears that tumor cells overexpress VEGF receptor, flt-1 and specific integrin isotype, such as $\alpha 6\beta 4$, are responders. This part of the work is currently pursued in Dr. Mitch Sokoloff's lab with addition of radiation and squalamine as a new combination. This work will be further explored and will be the subject of a future human clinical trial.

References:

None

Appendix:

1. Jin F, Xie ZH, Kuo CJ, Chung LWK and Hsieh CH. Co-target tumor and tumor endothelium effectively inhibits the growth of human prostate cancer by adenovirus-mediated antiangiogenesis and oncolysis combination therapy. *Cancer Gene Therapy* in press, 2005.
2. Chung LWK, Baseman A, Assikis V and Zhau HYE. Molecular insights into prostate cancer progression: The missing link of tumor microenvironment. *J. Urol.* in press, 2004.

Co-target Tumor and Tumor Endothelium Effectively Inhibits the Growth of Human Prostate Cancer by Adenovirus-mediated Antiangiogenesis and Oncolysis Combination Therapy

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Running Title: Co-targeting tumor and endothelium gene therapy

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Abstract

Tumor-endothelial interaction contributes to local prostate tumor growth and distant metastasis. A novel modality for the treatment of androgen-independent (AI) prostate cancer was designed to target not only the cancer cells but also the "crosstalk" between metastatic cancer cells and their surrounding microvascular endothelium. We have evaluated the synergistic and/or additive effects in antiangiogenesis as well as in inhibition of cancer cell growth by combination therapy of conditional oncolytic adenovirus with adenovirus-mediated antiangiogenesis both in vitro and in vivo. In the *in vitro* study demonstrated that human umbilical vein endothelial cells (HUVEC) and human AI prostate cancer cells, C4-2, infected with an antiangiogenic Ad-Flk-1-fc vector secreted a soluble form of Flk-1 which dramatically inhibited the proliferation, migration and tube formation of endothelial cells. C4-2 cells showed maximal growth inhibition when co-infected with Ad-Flk-1-fc and Ad-hOC-E1, a conditional replication-competent adenoviral (Ad) vector targeting both prostate cancer epithelial and stromal cells. Using a 3-dimentional (3D) co-culture model, we found that targeting C4-2 cells with Ad-hOC-E1 could markedly decrease tube formation in HUVEC, as visualized by confocal microscopy. In a subcutaneous C4-2 tumor xenograft model, tumor volume was decreased by 40~60% in animals treated with Ad-Flk-1-fc or Ad-hOC-E1 plus vitamin D_3 alone and 90% in combined treatment group, compared to untreated animals in an 8-week treatment period. Moreover, 3 of 10 (30%) pre-established tumors were completely regressed when animals received combination therapy. This co-target tumor and tumor endothelium could be a promising gene therapy strategy for the treatment of both localized and metastatic human prostate cancer.

Introduction

Hormone-refractory prostate cancer is one of the leading causes of cancer mortality and morbidity in North American men (1). Despite aggressive efforts toward earlier detection and treatment, the mortality rate for prostate cancer has steadily increased. The most common site of prostate cancer metastasis is the bone with skeletal metastases identified at autopsy in up to 90% of patients dying from prostate cancer (2, 3). While localized prostate cancer may be cured, patients with hormonal refractory and bone metastasis resulting complications often have a poor prognosis, with a medium survival of 9 months or less (4). Conventional therapy such as hormone therapy, radiation therapy and chemotherapy assumes that cancer is a clonal cell disease and targeting tumor epithelium can best control that tumor growth. These forms of therapy are highly effective but unfortunately not long lasting, and patients eventually become refractory to these therapeutic intervention. It is medical urgent to develop new therapy to circumvent this problem.

The growth and metastasis of prostate cancer cells are intimately affected by their microenvironment because of the well-established autocrine, paracrine and endocrine communication loops existing between cancer cells and their adjacent cell components (5), including 1) smooth muscle cells and fibroblasts, which provide critical soluble growth factors, and ECMs that support tumor growth and anchorage-dependent survival; 2) endothelial cells which form critical blood vessels, supply oxygen and nutrients to the tumor epithelium, and remove metabolic wastes from the tumor cells; 3) inflammatory cells which could support important cytokines to defend bacterial infection of the tumor epithelium and also potentially maintain the growth requirements of tumor cells. Prostate cancer therefore is considered as an organ not a single cell disease, Using experimental coculture cell model and chimeric tumor

model (6) comprising human prostate cancer and bone stromal cells, our laboratory proved a principle that cotargeting both tumor and its supporting stroma is more efficacious than targeting a single cell compartment of human prostate cancer bone metastasis. Recent studies co-targeting bone and tumor cells in clinical trails of hormone refractory prostate cancer using the combination of chemotherapy (which targets prostate tumor epithelium) plus strontium 89 (which targets bone), and an experimental model of prostate cancer skeletal metastasis using a conditional replication-competent adenoviral vector with viral replication controlled by a tissue-specific and tumor restrictive promoter, osteocalcin (OC), have been shown great promise by substantially improving patient survival (4) and curing animals with pre-existing prostate tumor in the skeleton (7). These findings further support that such cotargeting strategy may ultimately lead to the rational design of novel and effective modalities for the treatment of hormone-refractory human prostate cancer and bone metastasis.

Extensive studies by many investigators established that tumor-associated angiogenesis has a central role in the invasion, growth and metastasis of solid tumors (8) and can be targeted as a anticancer therapeutic strategy (9-11). Tumor cells influence this proves by producing angiogenic stimulators and inhibitors (12) through paracrine-mediated regulation. As a strategy for cancer therapy, antiangiogenic therapy attempts to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass (13). One antiangiogenic approach is to target the upregulated surface receptors on tumor endothelial cells (14). Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR2, also known as Flk-1) that binds the five isoforms of VEGF has a more restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature (15). Flk-1 has been the subject of numerous studies in

both animal models and in clinical trials (16-18). Unfortunately, the clinical efficacy of antiangiogenic therapy when used as single-agent therapy has been disappointing.

To advance the co-targeting of both tumor and tumor microenvironment, in the present study we proposed to evaluated the responsiveness of prostate cancer to conditional replication-competent adenovirus and VEGF receptor-based antiangiogenic therapy to target three cell components: prostate tumor cells, prostate and bone stromal cells and neovascular endothelial cells in both cell culture and experimental animal model. Our data demonstrated that adenovirus-meditated Flk-1-fc fusion protein delivery effectively suppresses angiogenesis and tumor growth through autocrine and/or paracrine mechanism. Targeting prostate cancer cells with conditional oncolytic adenoviruses obtains a bystander effect in antiangiogenesis. Moreover, a synergistic antitumor efficacy can be achieved when animals receive combination therapy.

Materials and Methods

Cell lines and Cultures

C4-2, an AI and metastatic human prostate cancer cell line derived from LNCaP (19) was grown in T medium (Invitrogen, CA) with 5% fetal bovine serum (FBS). HUVEC, a human umbilical vein endothelial cell line was obtained from Cambrex Bio Science (Cambrex, CA) and maintained in endothelium-specific medium (EGM-2, Cambrex) according to the manufacture's instruction. The cells were fed three times per week with fresh growth medium and maintained at 37°C in 5% CO_2.

A 3D co-culture system was established to assess the effect prostate cancer cells on endothelial tube formation. A GFP-tagged C4-2 cell line (C4-2-GFP) was generated by infection of C4-2 with a recombinant retroviruses containing a enhanced green fluorescence gene (EGFP) driven by a retroviral long terminal repeat (LTR) promoter. C4-2-GFP clones were obtained after selection of the transduced C4-2 cells by G418 (0.8mg/ml). HUVEC cells were labeled with a red-fluorescent lipid dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), according to the manufacture's instruction (Molecular Probes, OR). DiI-labeled HUVEC cells ($5x10^4$ cells/well) were plated on chamber slides, which were pre-coated with Matrigel (Becton-Dickinson, NJ). The Matrigel used in this experiment was diluted 1:1 in T-medium. Cells were allowed to settle for 1hr and then medium were carefully aspirated and replaced with 50μl of Matrigel. After polymerization (about 40 min), an overlay Matrigel containing $5x10^4$ C4-2/GFP cells preinfected with Ad-hOC-E1 or Ad-CMV-pA was added. Cell cultures were grown in EGM-2 plus T-medium with a ratio of 1:1 for 3 days.

Adenoviral vectors

Ad-Flk1-fc, an adenoviral (Ad) vector containing murine Flk-1-Fc signal peptide followed by the ectodomain of murine Flk-1 fused to the Fc fragment of murine IgG2a, and Ad-fc caring only the Fc fragment (20) were kindly provided by Dr. Kuo at Stanford University (Stanford, CA). Replication-competent Ad-hOC-E1 and its replication-defective control Ad-CMV-pA was constructed by our laboratory as described previously (21). All of the Ad vectors were amplified and purified according to the method of Graham and Prevec (22). Viral titer was determined by plaque assay.

Preparation of Conditioned Medium

$1x10^6$ C4-2 and HUVEC was seeded in 60-mm culture dishes overnight and then subjected to infection with PBS (mock injection), or 10 moi of Ad-Flk1-Fc or Ad-Fc. After 2 hr absorption, the virus was removed, and cells were cultured in fresh medium. After 48 hr culture, the conditioned medium (CM) of HUVEC and C4-2 cells was harvested and concentrated by ultrafiltration with a Centricon YA10 (Millipore, MA). The protein amount in CM was determined using BCA Protein Assay kit (Pierce, IL) and then stored in -80°C until used.

Western blot

Twenty μg of CM or 2μl of mouse plasma was used for immunoblotting using the NOVEX (Invitrogen, CA) system. Membrane was probed with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-murine IgG2a Fc antibody (Southern Biotech, AL). Immunoreactive bands were revealed by the enhanced chemiluminescent (ECL) plus system

(Amersham, NJ) according to the manufacture's instruction and quantified using Quantity one-4.1.1 Del Doc gel documentation software (Bio-Rad, CA).

Cell Proliferation and Cytotoxicity Assay

HUVEC cell proliferation assay was performed using the MTT method. Briefly, 4,000 HUVEC cells were seeded overnight on 96-well plates with EGM-2 medium containing 10ng/ml VEGF. Cells were subjected to infection with either Ad-Flk1-fc or Ad-fc at a moi of 10, or incubation with10 μg of concentrated CM from Ad-infected C4-2 for a period of 3 days followed by MTT assay. For *in vitro* cytotoxicity assay, $5x10^5$ C4-2 cells were seeded overnight on 24-well plate and then infected with 10 moi of Ad-Flk-1-fc or Ad-fc, and 2 moi of Ad-hOC-E1 or Ad-CMV-pA alone or combination. After 2 hr absorption, the viral-containing medium was replaced with fresh growth medium for additional 3 days culture. The relative cell number was assessed by optical density (OD) at 590 nm after crystal violet staining.

Tube Formation Assay

Tube formation on Metrigel was performed as described previously (23) with a minor modification. Briefly, the 24 well plates were coated with 200 μl Matrigel. HUVEC was trypsinized, washed and resuspended in EGM-2 medium in the presence or absence of CM (10 μg/ml) from Ad-infected C4-2. Cells (25,000 cells/100μl) were dispensed into each well and incubated for 8 hr. Each well was photographed under a phase-contrast microscopy at 40x magnification. The tube formation was quantified by counting the number of connecting branches between discrete endothelial cells.

Tubular structures generated in 3D coculture which was grown on chamber slides as described above were analyzed by a LSM 510 META laser scanning confocal microscopy (Carl Zeiss, NY). Images of serial optical sections were taken at 1μm thickness in a basal-to-apical direction using a x10 Neofluor objective.

Cell Migration Assay

HUVEC cell migration was performed using wound healing method (24). Briefly, Confluent monolayer of HUVEC grown on 24-well plates were wounded using a sterile 200-μl plastic pipette tip. Displaced cells were removed with three washes, and fresh EGM-2 medium containing C4-2 CM was added. Photographs were taken under phase-contrast microscopy at time 0, 12 hr and 24 hr after scraping. The position of wound edge was noted against a reference grid and the migrating distance was measured using Openlab 3.0.8 software (Improvision, MA). Data was presented as the means of 10 measurements at each time point.

Animal Studies

Five to eight-week-old male athymic mice (CD1 nu/nu) were purchased from Charles River (Wilmington, MA). The animals were kept under standard pathogen-free conditions and received care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* by the National Academy of Sciences. All animal experiments were approved by, and complied with the regulation of, the Emory University.

Xenografts were established by subcutaneously injecting $2x10^6$ C4-2 cells in 100μl Matrigel into the flanks of mice. Tumor-bearing mice were designated as small, medium and large tumor groups based on their established tumor volume: ~50mm^3, ~200mm^3 and ~500mm^3,

respectively. In each group, the mice were randomized and given PBS (vehicle control), $2x10^9$ pfu of Ad-Flk1-fc (intratumoral injection, twice per week for two weeks), or $2x10^9$ pfu of Ad-hOC-E1 (intravenous injection for single dose) alone or together. Mice receiving Ad-hOC-E1 were treated by i.p. administration of 100μl Vitamin D_3 (4ng/dose) every other day for 3 weeks. Vitamin D_3 treated mice were fed a sterilized calcium deficient diet (ICN Research Dies), as described previously (21). Tumor volume measurements were taken weekly and calculated according to the formula: length x width^2 x 0.5236. Data are expressed as fold of the end-point tumor volume in treatment groups relative to that in vehicle control group. Blood sample was also obtained from Ad-Flk-1-fc treated mice in small tumor group every other week by saphenous vein puncture with heparinized capillary tubes after anesthesia. All animals were sacrificed at 8 weeks after treatment. Tumors were excised and then either imbedded in OTC or fixed with 10% formalin.

Immunohistochemistry

For microvessel density analysis, snap-frozen tissues were sectioned into a 4-μm on a cryostat, fixed in cold acetone for 10 min and blocked with SuperBlock (Scytex Laboratories, UT) for 20 min, and then followed by incubation with a monoclonal rat anti-mouse CD31 at a 1:200 dilution (Chemicon, CA) at room temperature for 90 min. Sections were sequentially incubated with the secondary antibody, Alexa 594-conjugated goat anti-rat IgG at a 1:200 dilution (Molecular Probes, OR) at room temperature for 1 hr, and then mounted and examined under fluorescence microscopy. For TUNEL assay, paraffin-embedded tumor section was dewaxed and then subjected to cell permeabilisation by 350W microwave irradiation for 5 min. DNA strand break were then labeled with fluorescein-conjugated nucleotides in terminal deoxynucleotidyl

transferase reaction mixture supplemented in the *In Situ Cell Death Detection* kit (Roche, IN) at 37°C for 1 hr, and subjected to the reaction of anti-fluorescein-POD conjugate with diaminobenzidine according to the manufacture's instruction. The samples were counterstained with 1% methyl green to show viable cells.

Statistical analysis

Differences between treatment groups were analyzed using Student's *t* test and two-tailed distribution.

Results

Expression of soluble flk-1 on human endothelial and prostate cancer cell line by Ad-Flk1-fc

To test the antiangiogenic effect of VEGF receptor-based gene therapy approach in prostate cancer, a replication defective Ad vector, Ad-Flk-1-fc containing the VEGF receptor-2 (Flk-1) cDNA fused with a murine IgG2a Fc fragment (Fig. 1a) was used in this study. The gene transduction efficacy of Ad-Flk-1-fc in human prostate cancer cells (C4-2) and endothelial cells (HUVEC) was determined by Western blot of the secreted Flk-1 protein in the CM from Ad-Flk-1-fc-infected cells. Both HUVEC and C4-2 cells appeared to effectively produce Flk-1-fc fusion protein (185 kDa) or Fc (25 kDa) protein into CM when cells were infected with 10 m.o.i of Ad-Flk-1-fc or control vector Ad-fc, respectively (Fig. 1b). Quantification of protein expression shown in Western blot demonstrated a 6-fold higher susceptibility of HUVEC to Ad infection in comparison with C4-2 cells, which may due to the higher level of CAR (25) and integrin receptor expressed on the cell surface of HUVEC (data not shown).

Biological Effect of soluble Flk-1 on angiogenesis by Ad-Flk-1-fc in vitro

To evaluate the autocine and paracrine effect of Ad-Flk1-fc in tumor vasculature *in vitro*, HUVEC proliferation, migration and tube formation were assessed when cells were exposed to adenoviruses and/or CM from Ad-infected C4-2 cells. As shown in Fig. 2a, direct infection of HUVEC with 10 moi of Ad-Flk-1-fc inhibited cell proliferation by 25% compared with mock-treated cells. Massively inhibitory effect (65%) on proliferation of HUVEC was observed when cells incubated with CM from Ad-Flk-1-fc infected C4-2. Whereas no difference was seem among mock-treated, Ad-fc-infected and Ad-fc-infected C4-2 CM-treated cells. The paracrine effect of secreted Flk1 by Ad-Flk-1-fc-infected C4-2 cells on migration of HUVEC was also

determined by wound healing assay. The result demonstrated that HUVEC cells incubated with CM from C4-2 cells infected with either vehicle control (PBS) or Ad-fc can be seen to move toward and fill in a ~150-μm-wide cell free zone in a time-dependent manner. Similar effects were seen in the presence of CM from Ad-fc-infected C4-2, whereas CM from Ad-Flk-1-fc-infected C4-2 was able to abolish movement of HUVEC over a 24-h incubation period (Figs. 2b, 2c). In addition to proliferation and migration assays that describe the initial steps of endothelial cell activation, the ability of HUVEC cells to form tube-like structures on a Matrigel membrane in culture dishes was markedly reduced upon the additional CM from Ad-Flk-1-fc- but not Ad-fc-infected C4-2 by morphogenic assay (Fig. 2d). Quantitative analysis showed that Ad-Flk-1-fc inhibited tube formation of HUVEC by 50% compared to the control (Fig. 2e).

Inhibition of prostate cancer cell growth by Ad-Flk-1-fc in vitro

Recently, a potential autocrine role for VEFG in prostate cancer has been reported (26). To evaluate whether blocking the autocrine-mediated VEGF pathway could inhibit prostate cancer cell growth, we have firstly confirmed the expression of VEGF (736 pg/ml) and its type II receptor, Flk-1 in prostate cancer C4-2 cells by ELISA and RT-PCR (data not shown), respectively, and then cell proliferation of C4-2 cells infected with antiangiogenic Ad-Flk-1-fc, replication-competent Ad-hOC-E1, and their paired vector control, Ad-fc and Ad-CMV-pA (construct see Fig. 1a), respectively alone or together was determined and compared with that of mock-infected cells after 72 hr of incubation. As expectation, control vectors, Ad-fc and Ad-CMV-pA barely inhibited C4-2 growth but Ad-hOC-E1 effectively replicated and lysed C4-2 cells ($p<0.005$) when compared with mock-infection. Interestingly, Ad-Flk1-fc also suppressed cell growth significantly ($p<0.05$). Treatment of C4-2 with Ad-Flk1-fc and Ad-hOC-E1 together

enhanced cell number reduction even more dramatically when compared with Ad-Flk1-fc or Ad-hOC-E1 alone. These results demonstrated that Ad-Flk1-fc directly target not only angiogenesis but also prostate cancer cell growth and a potential additive effect of Ad-Flk1-fc on prostate cancer cell death when combining with other modality, such as oncolytic Ad vector.

"Bystander" antiangiogenesis by Ad-hOC-E1-mediated cell-kill of prostate cancer cells in vitro

Studies have demonstrated that the expression of angiogenesis-stimulating factors such as VEFG, platelet-derived growth factor, and transforming growth factor in prostate carcinoma is increased (27-29), which suggests a possible role of prostate cancer on endothelial angiogenesis. To determine if the stimulatory effect of prostate cancer cells on endothelial vasculature is blocked by the elimination of cancer cells, tumor-driven tube formation of endothelial cells was conducted in a 3D co-culture model in which C4-2-GFP and DiI-labeled HUVEC cells grew separately on two layers of Matrigel, followed by confocal imaging. As expected, HUVEC cultured in 3D Matrigel organized into an irregularly vasculogenic-like network with a total branch extension of 85±7μm by confocal microscopy analysis (Fig. 4a). The distance of network formed by HUVEC was increased upon coculture of HUVEC with C4-2 cells (450±71μm) demonstrating the inductive role of prostate cancer cells on tumor vasculature. Preinfection of C4-2 cells with Ad-hOC-E1, which has been demonstrated to selectively replicate in OC-expressing but not non-OC-expressing cells (21), we observed not only a decreased growth of C4-2 but also a reduced network formation by HUVEC (175±3.5μm) in coculture (Fig. 4c), whereas no difference was seem in HUVEC cocultured with Ad-fc pre-infected C4-2 (Fig. 4b) compared with parental C4-2. Taken together, these results evidenced that, for the first time, the prostate cancer-induced vasculogenesis of endothelial cells, and demonstrated a "bystander"

antiangiogenic effect of Ad-hOC-E1 on endothelial cells (non-OC-expressing cells) by the elimination of their supporting prostate cancer cells (OC-expressing cells).

Enhanced antitumor efficacy by Ad-Flk-1-fc and Ad-hOC-E1 plus Vitamin D_3 combination therapy

We evaluated the antitumor effect of Ad-Flk1-fc, Ad-hOC-E1 plus vitamin D_3 alone or together in subcutaneous C4-2 tumors in athymic mice. To determine whether adenovirus-encoded Flk-1-fc can be efficiently expressed by pre-existing prostate tumors and secreted into blood circulation over an extended period using an intratumoral gene delivery approach, we analyzed plasma samples from tumor-bearing mice that had received intratumoral injection of $2x10^9$ pfu of Ad-Flk-1-fc twice per week for 2 weeks. Blood samples from individual mice (Fig. 5a) or from the same mice at different time points (Fig. 5b) were analyzed. High levels of Flk-1-fc were found in the majority of mouse plasma from Ad-Flk-1-fc-treated animal at 1 day after the last administration and prolonged for over 6 weeks. These data demonstrated that high level of Flk-1-fc in the mouse blood circulation was achieved by adenoviral vector-mediated intratumoral gene delivery. To investigate the influence of tumor burden in response to the treatment, tumor-bearing mice were designated as small, medium and large tumor groups based on the established tumor volume: ~50mm^3, ~200mm^3 and ~500mm^3, respectively prior to treatment. C4-2 tumor xenografts were treated with intratumoral Ad-Flk1-fc and intravenous Ad-hOC-E1 either alone or together, or PBS. Ad-hOC-E1-treated mice were given intraperitoneal Vitamin D_3 to augment oncolytic activity of Ad-hOC-E1 according to the pre-established protocol (21). As shown in Fig. 5c, both Ad-Flk1-fc and Ad-hOC-E1 plus vitamin D_3 alone slightly reduced C4-2 tumor growth in large and medium tumor group, but the change is not statistically significant ($p>0.05$),

In contrast, combination therapy by Ad-Flk1-fc and Ad-hOC-E1 plus vitamin D_3 markedly inhibited C4-2 tumor growth with a 40~60% size reduction in large and medium tumor groups ($p<0.005$). Moreover, in small tumor group, despite the statistically significant tumor regression ($p<0.05$ in Ad-hOC-E1 plus vitamin D_3 and $p<0.005$ in Ad-Flk-1-fc and combination therapy) was seen in three treatment groups, 3 of 10 (30%) pre-established tumors were completely regressed only when animals received combination therapy.

Inhibition of angiogenesis and induction of tumor cell apoptosis in vivo

To determine whether the reduction in tumor growth was associated with a corresponding reduction in vascular density in the tumors, representative tumors receiving different treatment in the small tumor group were sectioned for immunohistochemistry at the end of treatment. Blood vessels in the tumor sections were identified by immunostaining using antibodies to the endothelial cell marker CD31. C4-2 tumors from animals receiving PBS showed intense staining for CD31 (Fig. 6a), indicating the presence of extensive angiogenesis in this type of tumor. Significantly reduced CD31-stained vessels was demonstrated after therapy with Ad-hOC-E1 plus vitamin D_3, but the effect was much more remarkable with Ad-Flk-1-fc, particularly when used in combination with Ad-hOC-E1 (Fig. 6a). Quantification of the results reveals a reduction in microvessel density of 20%, 50% and 70% in tumors treated by Ad-hOC-E1 plus vitamin D_3 and Ad-Flk-1-fc alone and together, respectively, in comparison with the vehicle control mice (Fig. 6b). Furthermore, TUNEL staining of tumor sections from different treatment groups was performed to demonstrate the induction of apoptosis with these therapeutic approaches. As shown in Fig. 6c, apoptotic cells were seldom seen in tumors from vehicle control mice but occasional apoptotic tumor cells were evident after Ad-hOC-E1 plus vitamin D_3 treatment. In

contrast, numerous intense staining was observed in tumors receiving Ad-Flk-1-Fc alone, and combination therapy led to a progressive increase in cell apoptosis compared to Ad-hOC-E1 plus vitamin D_3 or Ad-Flk-1-fc treatment alone. These results suggest that treatment of C4-2 tumors with Ad-hOC-E1 plus vitamin D_3 and Ad-Flk-1-Fc together advanced both tumor vasculature regression and tumor cell death, which is attributable to the insufficient cell-cell interaction between tumor and tumor endothelium.

Discussion

As most common solid cancers frequently mutate in response to therapy, effects have been made to eradicate tumors by therapies directed against the tumor microenvironment. The inhibition of tumor growth by attacking the tumor's vascular supply offers a primary target for antiangiogenic intervention. However, result from our (Fig. 5b) and other laboratories (30) demonstrated that targeting a single pro-angiogenic molecule might be associated with eventual resistance to therapy when tumor cells accumulate other pro-angiogenic molecules production with increased tumor burdens. Here we developed a novel strategy that might overcome problems of tumor-cell heterogeneity (tumor cell-specific targeting) and the redundancy of tumor-associated angiogenesis-stimulating factors (tumor endothelium-specific targeting). We accomplished this by exploiting the obvious advantages of conditional replication-competent adenoviruses and antiantiogenic therapies developed by us (7, 21) and other investigators (20, 31), respectively, based on the well-established reciprocal cellular interaction that occurs between prostate cancer, cancer-associated stroma and tumor endothelium ultimately cascades into the cancer growth, progression and metastasis (5, 32). Results from our studies provide further evidence in support of the view that cotargeting tumor cells and tumor microenvironment is more efficacious than targeting a single cell compartment.

Compared with other strategies currently under clinical investigation that using either small molecule inhibitors or monoclonal antibody-based antiangiogenic agents in combination with traditional antitumor drugs {Reviewed by Retter *et al.* 2003 (33)}, the advantage of our strategy which combines adenovirus-mediated antiangiogenesis with oncolytic adenoviruses and vitamin D_3 for the treatment of prostate cancer is every therapeutic agent we chose for this combination has dual even multiple targets. We has previously demonstrated that OC promoter-

mediated oncolytic adenoviruses has a broad spectrum of cell kill activity that caused lysis in PSA-producing and –nonproducing prostate tumor, bone, and prostate stromal cells *in vitro* (7, 21). Vitamin D_3 used in animal study has been shown its antiproliferative effect on prostate cancer cells when used as single agent (34) and its capacity to enhance adenoviral replication when combined with Ad-hOC-E1 through an inductive role on hOC promoter (21). Moreover, the direct inhibitory effect on tumor-induced angiogenesis by vitamin D_3 was also demonstrated in several experimental studies (35-37). Adenoviral vector locally delivering a recombinant, soluble Flk-1 receptor into both tumor epithelial and endothelial cells is capable to regress tumors through not only "starving" the tumor cells by blocking tumor vasculatures but also through the direct cell-killing effect on the tumor cells themselves, as demonstrated for the first time in the present study (Fig. 3). This finding is consistent to the previous report (26) that stimulate of LNCaP cells with recombinant VEGF induces DNA synthesis and recruits quiescent cells into the S-phase of the cell cycle via signaling through Flk-1. Moreover, as hypothesized by other investigators (38, 39), the inability of replication-defective Ad-Flk-1-fc to diffuse through tissues may be improved by acquiring ability to multiply via the exogenous adenoviral E1 gene product provided by Ad-hOC-E1 within tumors in our combination therapy approach, which can also magnify the antitumor efficacy from Ad-Flk-1-fc. Combining Ad-hOC-E1, vitamin D_3 and Ad-Flk-1-fc therefore represents an attractive therapeutic approach to prostate cancer.

Unlike small molecule inhibitors or anti-VEGF monoclonal antibodies, which are required for prolonged administration to achieve a sufficient long-term steady state level of the protein, a single intravaneous injection of Ad vector expressing a secretable antiangiogenic protein is sufficient to express persistent levels of gene products in mouse blood circulation (20, 31, 40). However, the utility of systemic gene delivery by Ad vector is limited because of the

hepatotoxicity resulting from overexpression of antiangiogenic factors in hepatocytes by localizing Ad vectors primarily to the liver (41). In our model system, local, intratumoral injection of Ad-Flk-1-fc achieves both local and systemic accumulation of the Flk-1-fc at least for 6 weeks, which results in sufficient suppression of neovascularization in C4-2 tumors via autocrine and/or paracrine actions with no observed organ toxicity. Our experiments provide evidence supporting the advantage of using Ad vectors for regional, *in vivo* antiangiogenic prostate cancer gene therapy. However, several issues remain to be addressed, and some of them cannot be examined using currently available animal models. The host range of human adenoviruses is restricted, and no appropriate animal model exist to allow further exploration of the effectiveness of adenoviral vectors in immune-competent animal models. In a realistic scenario, intratumoral administration of the adenovirus in cancer patients, particularly after multiple treatment, may result in the production of neutralizing antibodies with subsequent elimination of infected cells, however, additional virus-induced cytotoxic effect might be beneficial. Thus, an immune response directed against viral antigens might augment tumor killing by affecting non-infected tumor cells. In addition, the intratumoral recruitment and stimulation of tumor-specific T lymphocytes theoretically could lead, in some cases, to systemic anti-tumor immunity.

Experimental data have provided intriguing evidence that aggressive breast and prostatic carcinoma and melanoma tumors demonstrate vasculogenic mimicry (42-44) by the ability of tumor cells to form *de novo* vasculogenic-lik networks *in vitro* in the absence of endothelial cells or fibroblasts, concomitant with the expression of several vascular-associated markers, including thrombin receptor, endothelin-B receptor, endoglin, TIE-2 or Flk-1 (45). Other than *in vitro* cell models, tumor cell-lined vasculature is detectable in clinic specimen (46, 47) suggesting a

positive role of vasculogenic mimicry in established, growth, and metastasis of aggressive human tumors. We have the similar observation in prostate cancer that the ability of prostate cancer cell lines to form patterned vasculogenic-like networks in 3D Matrigel culture *in vitro* is associated with their tumorigenecicy *in vivo* (Jin's unpublished data). Additional molecular studies are in progress in our laboratory to elucidate the molecular mechanism responsible for the vasculogenic mimicry. Based on the expression of Flk-1, one of the key vascular markers in C4-2 cells, it is tempting to speculate that the induction of tumor cell apoptosis by Ad-Flk-1-fc shown in our study may be attributable to, in part, block the tumor-lined vasculature (in addition to endothelial-lined vasculature).

In summary, we have demonstrate that adenovirus-mediated intratumoral transfer of a ectodomain of Flk-1 gene to be an efficient means of delivering the gene to both vascular endothelial cells and prostate cancer cells resulting in suppressed tumor growth via an indirect antiangiogenic mechanism and/or direct tumor-cell kill. Eliminating cancer cells by a conditional replication-competent adenovirus has a bystander effect on tumor-induced angiogenesis. Combining antiangiogenesis and oncolytic adenoviruses gene therapy approach achieved a synergistic effect in elimination of prostate tumor growth in an experimental model. Our "proof-(21)of-concept" study establishes for the first time that adenovirus-mediated co-targeting prostate cancer and tumor endothelium may be an effective strategy for destroying AI and metastatic human prostate tumors.

Acknowledgments

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Figure legends

FIG. 1. (a) Organization of the vectors used in this study. An E1- and E3-deleted antiangiogenic Ad5 vector, Ad-Flk-1-fc, caring a murine Flk1 cDNA sequence encoding the signal peptide and the ectodomain (Mu Flk1 Ec) fused to a murine IgG2a Fc fragment, and its vector control construct, Ad-Fc were controlled by human cytomegalovirus (CMV) promoter and the rabbit β-globin intron and polyadenylation signal (pA). In a replication-competent Ad vector Ad-hOC-E1, Ad5 E1A and E1B expression are driven by bidirectional hOC enhancer/promoter (E/P) and terminated by the SV40 pA. A replication-defective Ad-CMV-pA was served as vector control of Ad-hOC-E1. (b) Western blot analysis of Flk-1 expression. 20μg of concentrated CM from HUVEC and C4-2 cells infected with Ad-Flk1-fc, control vector Ad-fc, or mock infection (PBS) was subjected to Western blot using polyclonal antibody recognizing murine IgG2a Fc as described under Materials and Methods. Soluble Flk1-fc and fc was detected at MW of 185 and 25 kDa in CM from Ad vector-infected cells.

FIG. 2. Biological effects of adenovirus-mediated soluble Flk1 expression on HUVEC cells. (a) Inhibition of HUVEC proliferation by Ad-Flk1-fc. HUVEC cells were infected with Ad-Flk1-fc or Ad-fc at m.o.i. of 10 or incubated with CM from C4-2 cells (C4-2 CM) infected with Ad-Flk1-fc or Ad-fc at a final concentration of 10μg/ml for 72 hr. Cell proliferation was determined by MTT assay. Data are given as means of absorbance at 575 nm and SD of four independent experiments; *versus* mock-infected group: *, $p<0.05$; **, $p<0.005$. (b, c) Inhibition of HUVEC migration by Ad-Flk1-fc. HUVEC migration was determined in a wound healing assay as described in Materials and Methods. (b) Representative photomicrographs of vehicle-treated

control cells (PBS) and cells treated with CM from C4-2 cells, which were infected with Ad-Flk1-fc (Flk-fc) or Ad-fc (fc) at the indicated time point are shown (25X magnification). (c) Quantitative measurement of cell migration. Data are expressed as mean ± SD of three independent experiments, *verses* PBS control: $^{**}p<0.005$. (d, e) Inhibition of HUVEC tube formation by soluble Flk1 expressed by Ad vector. (d) Representative photomicrograph of HUVEC incubated with CM as described above on Matrigel-coated well for 72 hr was shown (40X magnification). (e) The tube formations were quantified by counting the number of connecting branches between two discrete endothelial cells. Data are given as means ± SD of four independent experiments, *versus* mock-infected group: $^{**}P < 0.005$.

FIG. 3. Cytotoxicity of C4-2 cells by Ad vectors *in vitro*. C4-2 cells were infected with PBS (mock infection), Ad-Flk-1-fc (10 moi), Ad-fc (10 moi), Ad-hOC-E1 (2 moi) and Ad-CMV-pA (2 moi) alone or Ad-Flk-1-fc plus Ad-hOC-E1 (combination). The cytotoxicity assay was performed using crystal violet staining and the relative cell number was assessed by absorbance at 590 nm after staining: *versus* mock-infected group: *, $p<0.001$; **, $p<0.0001$; *versus* Ad-Flk-1-fc or Ad-hOC-E1 group: # $p<0.05$.

FIG. 4. "Bystander" anti-vasculogenesis of endothelial cells by Ad-hOC-E1-infected AI prostate cancer cells in a 3D Matrigel coculture system. Representative photograph showing the vessel network formed from the culture of (a) DiI-labeled HUVEC cells alone, or cocultured DiI-labeled HUVEC with (b) Ad-fc or (c) Ad-Flk-1-fc pre-infected C4-2-GFP cells in Matrigel at Day 3 using a laser scanning confocal microscopy (100X magnification). Individual images are labeled with the indicated distance from the bottom of the HUVEC grown.

FIG. 5. Animal studies of combination therapy with Ad-Flk1-fc and Ad-hOC-E1. (a, b) Detection of Flk-1 in Ad-Flk-1-fc-treated mice plasma by Western blot analysis. (a) Tumor-bearing mice (#1 to #5) were treated with Ad-Flk-1-fc intratumorally twice per week for 2 weeks and the plasma levels of Flk-1-fc was detected before the 1st treatment or at the 1st week after the last treatment. 20μg of CM from Ad-Flk-1-fc infected HUVEC cells (CM) as described in Materials and Methods was served as positive control. (b) Representative plasma levels of Flk-1 from individual Ad-Flk-1-fc-treated mice (#2) before the 1st treatment or after the final treatment at the indicated times. (c) Antitumor efficacy of Ad-Flk1-fc, Ad-hOC-E1, or combination on C4-2 tumor xenografts in nude mice. Three groups of mice bearing small, medium and large tumor burdens were treated with Ad-Flk1-fc, Ad-hOC-E1, either alone or together (combination), or PBS as control for an 8-weeks treatment protocol. Size of tumor at the endpoint was measured and presented as mean of percentage of control tumor and SD in each group, n=8-10. *<0.05 compared with the control-treated tumors.

FIG. 6. Inhibition of tumor angiogenesis and increase of tumor cell death by treatment with Ad vectors. Tumor-bearing mice in small tumor group were killed at the end of treatment protocol and tumor tissue was removed. Microvessel density was determined by anti-CD31 staining. (a) Representative photomicrograph showing microvessels in tumors from animals receiving the indicated treatment (100X magnification). (b) Quantitative analysis of microvessel density was made by counting the positive stained cells in 10 high power fields (HPF, x400 magnification). Data are given as mean±SD of cell number/HPF. *, $p<0.005$ compared with the control group receiving PBS; #, $p<0.05$ compared with either Ad-hOC-E1/vit. D_3 or Ad-Flk-1-fc treatment

group. (c) Representative photomicrograph of TUNEL staining showing increase of apoptotic cells (arrow indicated) within tumor tissues by treatment with Ad vectors (200X magnification).

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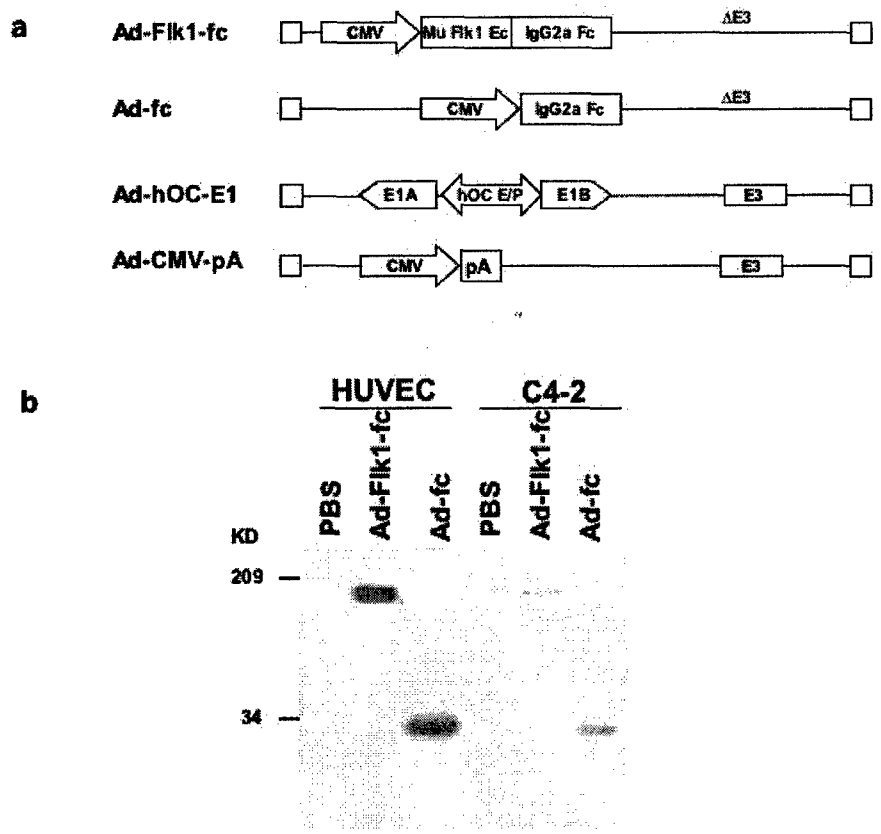
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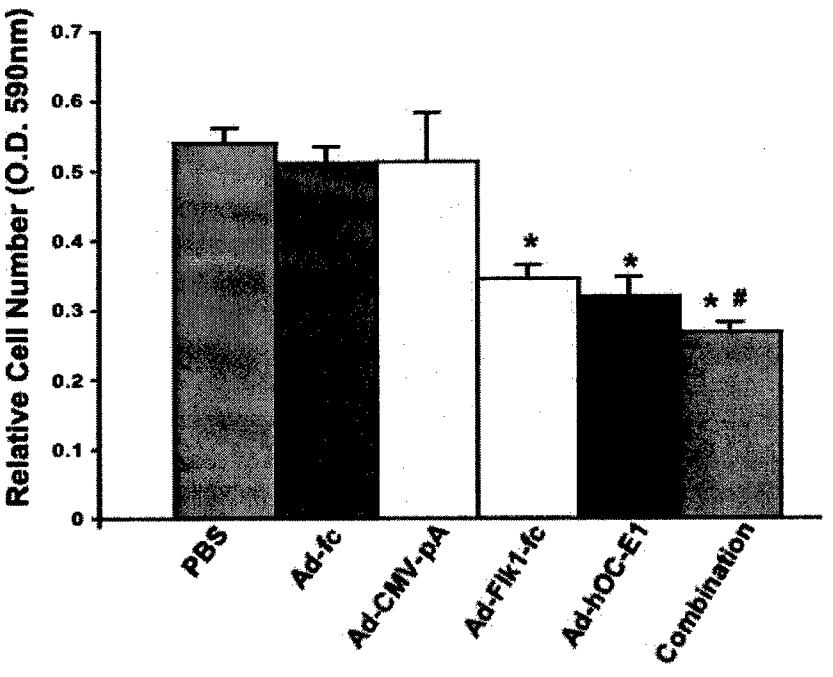
Figure 1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Ad-Flk1-fc
Ad-hOC-E1
Combination

Figure 3

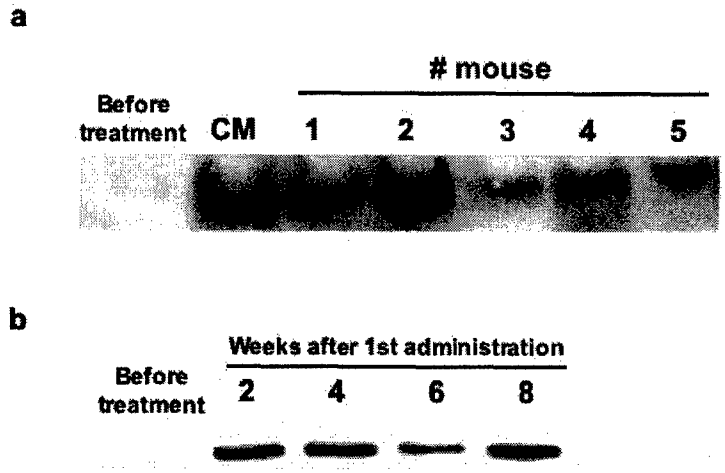
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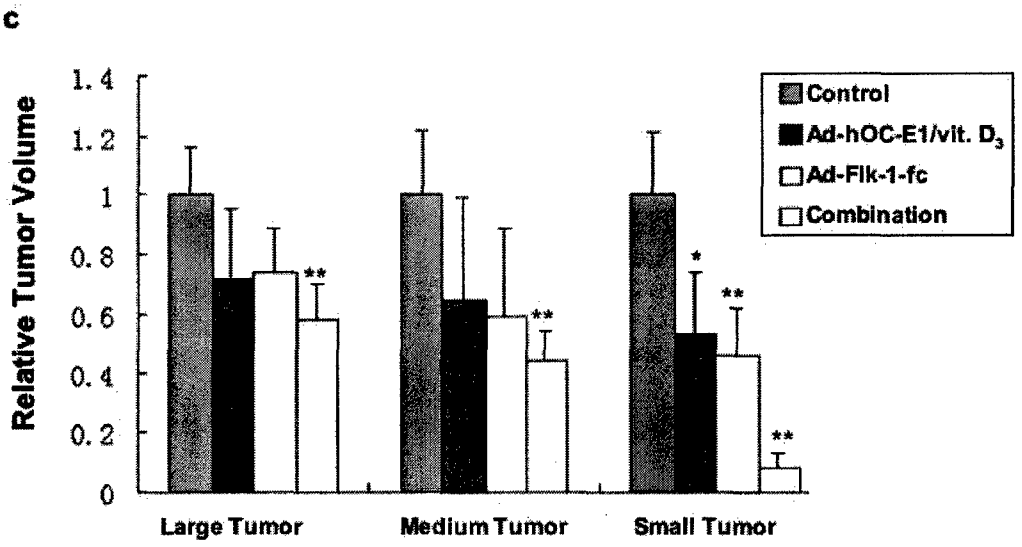
Figure 5a, b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Ad-hOC-E1/vit. D3
Ad-Flk-1-fc
Combination
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Large Tumor
Medium Tumor
Small Tumor

Figure 5c

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Abstract: Abstract
Purpose: Tumor cell genotype and phenotype have been considered as the sole determinants supporting cancer growth and metastasis. This review focuses on the published literature that suggests tumor-microenvironment interaction plays a decisive role in controlling local cancer growth, invasion and distant metastasis. As this review shows, genetic alterations in prostate cancer cells alone are not enough to confer metastatic status without a supporting tumor microenvironment. Effective therapeutic targeting requires a deeper understanding of the interplay between tumor and stroma. Approaches co-targeting tumor and stroma already show promise over the conventional targeting of tumor cells alone in preventing prostate cancer progression and eradicating pre-existing or newly developed prostate cancers in bone and visceral organs.

Materials and Methods: A literature survey using the MEDLINE database was performed in both basic and clinical publications relevant to tumor-host microenvironment interaction. Information pertinent to the biology and therapy of prostate cancer local growth and distant metastases was specifically emphasized.

Results: Tumor-associated stroma actively fuel the progression of prostate cancer from localized growth to invasion of surrounding tissues and the development of distant bone and visceral organ metastasis. In concert with this progression, tumor cells recovered from metastatic sites could either represent a subpopulation of pre-existing tumor cells or could be a newly acquired variant subsequent to tumor-stroma interaction. Experimental data from our laboratory and others suggest that permanent genetic and phenotypic changes occurred in prostate cancer cells after 3-D co-culture in vitro or when co-inoculated and grown with inductive stromal cells in vivo. These results support the idea that newly acquired variants are the dominant mechanism of prostate cancer progression. Intercellular communication between prostate cancer cells and organ-specific stroma including prostate and marrow stroma could involve diffusible soluble and solid matrix molecules as mediators, leading to the development of metastasis. This presents a new opportunity for therapeutic targeting in the treatment of benign and malignant growth of the prostate glands. This review summarizes specific research implicating tumor-microenvironment interaction as the molecular basis of cancer progression, providing a rationale for targeting both tumor and tumor-associated microenvironment in the management of androgen-independent and bone metastatic prostate cancer progression in patients.

Conclusions: Cancer is not a single-cell disease. Both the aberrant cancer cells and their interactive microenvironment are needed for prostate cancer to progress to androgen independence and distant metastasis. It is highly plausible that newly evolved prostate cancer cell clones dominate cancer metastasis after cell-cell and cell-matrix interaction with host microenvironment [rather than the selection or expansion of a pre-existing prostate cancer cell clone(s)]. Based on this premise, potential molecular targets in the microenvironment are especially emphasized. Further elucidation of the molecular mechanisms underlying tumor-stroma interaction may yield improved medical treatments of prostate cancer growth and metastasis.

Molecular Insights into Prostate Cancer Progression: The Missing Link of Tumor Microenvironment

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Key words: tumor-stroma interaction; prostate; cancer progression; EMT or epithelial to mesenchymal transition; bone metastasis; osteomimicry; vasculogenic mimicry; reactive stroma; therapeutic targeting; growth factors; extracellular matrix; 3-D cell culture; cancer metastasis

Running title: Tumor-Stroma Interaction

Abbreviations: BSP, bone sialoprotein; ECM, extracellular matrix; EGF(R), Epidermal growth factor (receptor); EMT, epithelial to mesenchymal transition; ET-1, endothelin-1; FAK, focal adhesion kinase; HGF/SF, Hepatocyte growth factor/scatter factor; IGF-1, insulin-like growth factor-I ;IL-6, interleukin-6; ITT, intention to treat; MMP, matrix metalloproteinase; OC, osteocalcin; ON, osteonectin; OPN, osteopontin; PDGF(R), platelet derived growth factor (receptor); PI3K, phosphatidylinositol 3-kinase; TGF-β, transforming growth factor-β; TIP, tumor interstitial pressure; TTP, time to progression; VEGF, vascular endothelial growth factor.

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Abstract

Purpose: Tumor cell genotype and phenotype have been considered as the sole determinants supporting cancer growth and metastasis. This review focuses on the published literature that suggests tumor-microenvironment interaction plays a decisive role in controlling local cancer growth, invasion and distant metastasis. As this review shows, genetic alterations in prostate cancer cells alone are not enough to confer metastatic status without a supporting tumor microenvironment. Effective therapeutic targeting requires a deeper understanding of the interplay between tumor and stroma. Approaches co-targeting tumor and stroma already show promise over the conventional targeting of tumor cells alone in preventing prostate cancer progression and eradicating pre-existing or newly developed prostate cancers in bone and visceral organs.

Materials and Methods: A literature survey using the MEDLINE database was performed in both basic and clinical publications relevant to tumor-host microenvironment interaction. Information pertinent to the biology and therapy of prostate cancer local growth and distant metastases was specifically emphasized.

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D co-culture *in vitro* or when co-inoculated and grown with inductive stromal cells *in vivo*. These results support the idea that newly acquired variants are the dominant mechanism of prostate cancer progression. Intercellular communication between prostate cancer cells and organ-specific stroma including prostate and marrow stroma could involve diffusible soluble and solid matrix molecules as mediators, leading to the development of metastasis. This presents a new opportunity for therapeutic targeting in the treatment of benign and malignant growth of the prostate glands. This review summarizes specific research implicating tumor-microenvironment interaction as the molecular basis of cancer progression, providing a rationale for targeting both tumor and tumor-associated microenvironment in the management of androgen-independent and bone metastatic prostate cancer progression in patients.

Conclusion: Cancer is not a single-cell disease. Both the aberrant cancer cells and their interactive microenvironment are needed for prostate cancer to progress to androgen independence and distant metastasis. It is highly plausible that newly evolved prostate cancer cell clones dominate cancer metastasis after cell-cell and cell-matrix interaction with host microenvironment [rather than the selection or expansion of a pre-existing prostate cancer cell clone(s)]. Based on this premise, potential molecular targets in the microenvironment are especially emphasized. Further elucidation of the molecular mechanisms underlying tumor-stroma interaction may yield improved medical treatments of prostate cancer growth and metastasis.

Overview

Cancer cells reside in an organotypic host microenvironment which has long been under-emphasized because it is perceived only as a "silent bystander". Past understandings of the organ-specific profile of cancer and metastasis ("soil and seed") as originally proposed by Paget in 1889 (1) led to the idea that pre-existing subpopulations of cancer cells successfully complete a rather inefficient process called metastasis (2, 3). Strong experimental evidence suggests that primary tumors are heterogeneous and that subsequent observed metastasis is the result of a non-random, sequential, multi-step selective process among pre-existing cell subtypes (2, 4). Kauffman et al. (5) reviewed the roles of metastatic suppressor genes whose loss may prompt the selective growth and survival of cancer cells at certain secondary sites. Chambers et al. (6) suggested that molecular interaction between cancer cells and their metastatic organ site determines the success of cancer colonization. These selective processes are generally believed to occur rarely and during the late stages of tumor progression (3, 5). Hence this raises the question whether molecular profiling of cancer signatures at the primary prior to cancer metastasis can reliably predict clinical outcome (7, 8). A compromise idea was proposed by Kang et al. (9), who suggested that the expression of certain genes in primary breast cancer may indeed be prognostic but organ-specific tropism can be achieved only *after* the cancer cells have expressed a concrete set of overt bone-metastasis genes. In this understanding, tumor microenvironment is the "missing link" that not only provides fertile "soil" for cancer growth but also exerts dominant *inductive* influences that trigger permanent genetic and phenotypic changes in cancer cells, conferring their selective growth and survival advantages in subsequent dissemination. Thus, the characteristic metastatic cell clones to bone could have a loss of expression of metastatic suppressor genes, enhanced ability to interact with primary and

secondary organ sites, and acquisition of expression of a set of bone metastatic genes *after* cellular interaction with host microenvironment, either in the primary or at mestastatic sites, by the cancer cells.

The host microenvironment could participate actively in this rather inefficient and non-random metastatic process, in which cancer cell variants evolve after tumor-stroma interaction at primary or secondary sites of tumor growth. The molecular processes associated with this interaction are reviewed in several contexts: 1) The reciprocity of cancer cell-microenvironment interaction that facilitates the development of "osteomimicry" and "vasculogenic mimicry" by cancer cells (10). In other words, cancer cells can "mimic" the gene expression profiles of cells in their microenvironment. For instance, prostate cancer cells can express bone cell-like (osteomimetic) properties (10, 11) and melanoma can express vascular endothelial-specific markers in vasculogenic mimicry (12, 13); 2) The ability of cancer cells to undergo morphologic transitions, such as epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), is accompanied by the ability of cancer cells to acquire altered behaviors. Cancer cells observed to undergo EMT, often at the invasion front of a tumor, acquire increased motility, leading to migration and invasion (14-16); 3) There are coordinated and stable changes of gene expression profiles noted in cancer cells grown as 3-dimensional (3-D) organoids, with proliferation and survival intimately linked to the surrounding interface tissue matrix microenvironment (17-19). Perturbations in cancer-matrix interaction have been observed to alter important cell functions such as cell polarity, secretion, migration and invasion. Understanding the molecular mechanisms involved in the evolution of cancer cells and the reversibility and vulnerability of this process could help design future therapeutic agents. This review specifically considers the recent development of clinical trials targeting the tumor

microenvironment, such as bone stroma, tumor angiogenesis, paracrine signaling, host immune-directed and other co-targeting strategies for the treatment of prostate cancer and its metastasis.

Experimental models demonstrate that tumor microenvironment selectively enhances cancer progression

There are three commonly used methods to enhance the tumorigenic and metastatic potential of human prostate cancer cells. These are: 1) to stably transfect genetic materials to prostate cells that encode oncogenes, metastasis-associated genes, inactivating tumor suppressors, cell cycle regulators, and downstream mediators regulating cell proliferation, survival and apoptosis; 2) to deprive prostate cancer cells of androgen in cultured media or to grow prostate tumors in surgically castrated hosts; and 3) to co-inoculate prostate cancer cells with either relevant organ-specific stromal cells or tumor-derived extracellular matrices (20-22). Of these three approaches, androgen deprivation and tumor-stromal interaction seem to mimic best the natural history of prostate cancer progression in patients, i.e. the resulting tumor cells have the ability to undergo androgen-independent and bone metastatic progression. Unfortunately, the interplay between cancer cells and host stroma remains unclear because there is no transgenic animal model that specifically harbors a prostate cancer microenvironment defect, leaving us with a knowledge gap in defining the precise role of tumor microenvironment in cancer progression and metastasis§. Nevertheless, from the published data we can conclude that both the genetic make-up of a cancer cell and its host interaction shape the tumorigenic and metastatic potential of cancer cells. Since cancer growth and subsequent metastasis *in vivo* is possible *only* if the experiments are conducted in live animals, it is an inescapable conclusion that the host microenvironment must be involved even when genetically defined cells are tested.

Consistent with the above theme, it has been shown that orthotopic rather than ectopic inoculated prostate tumor cells acquire soft tissue and bone metastatic capability (23, 24). Permanently altered prostate cancer cell clones (phenotypic and genotypic) isolated after *in vivo* cellular interaction with bone stromal cells (25) or *in vitro* 3-D cellular interaction with prostate or bone-derived stromal cells acquired bone and soft tissue metastatic potential (26, 27). These results imply that an organ-specific stroma milieu comprised of different cell types which secrete different growth factors, extracellular matrices, metalloproteinases, and/or angiogenic molecules must be responsible for the process driving non-tumorigenic or non-metastatic prostate cancer cells to yield tumorigenic and metastatic phenotypes. Direct evidence that factors from host rather than tumor contribute to angiogenesis and tumor formation was provided by Huang et al (28) who showed that human ovarian cancer growth as peritoneal tumors and ascites was lower in transgenic immune-compromised nude mice lacking the MMP-9 gene due to the decreased level of ECM remodeling and angiogenesis adjacent to the sites of tumor colonization. They identified macrophages as the source of the MMP-9 supporting ovarian tumor growth, angiogenesis and spread. When they resolved the MMP-9 deficiency by transferring spleen cells as a source of macrophages to MMP-9 deficient mice, the growth, angiogenesis and colonization of ovarian tumors in the recipient mice was restored.

Results such as these support the exciting concept that stroma is a potential target for ovarian cancer treatment (29). Table 1 summarizes a number of well-characterized human prostate cancer lineage-related progression and xenograft models that could be used to study tumor-host microenvironment interaction. Among these models are the LNCaP progression model and the invasive ARCaP model established by our laboratory (24, 25). These models share important common features: they express two lethal phenotypes of human prostate cancer,

androgen-independence and bone metastasis; they consist of lineage-related cell lines evolved from the original cell clone, with common genetic backgrounds but diverse phenotype and behavior; the cells derived from both models express androgen receptor (AR) and secrete prostate-specific antigen (PSA); and they were both derived from *in vivo* tumors grown as 3-D xenografts under the influence of host factors and subjected to tumor-stroma interaction. *The most remarkable aspect of these models is that the prostate cancer cells acquired increased tumorigenic and metastatic potential merely through cellular interaction with the host microenvironment under 3-D conditions without requiring the transfer of any exogenous genes to the indolent-appearing cancer cell lines.* Control studies using similar cell types, either grown alone or with organ-specific stromal cells under 2-D conditions, failed to generate invasive and metastatic variants.

Intercellular communication between cancer cells and their surrounding stroma

Interactions between soluble factors and their receptors dictate gene expression profiles in both cancer cells and their surrounding stroma

Mediation of stromal-epithelial interactions in the normal and malignant prostatic environment involves a number of soluble factors and their receptors. Soluble factors can serve paracrine, autocrine or intracrine functions with their actions mediated by their respective receptors or interactive partners. Soluble factors could mediate in a reciprocal manner intercellular communication between stroma and epithelium that controls normal prostate development, benign enlargement of the prostate gland and its neoplastic transformation. The constellation of secreted soluble factors by different tissues may serve as chemoattractants or local growth inducers via appropriate cancer cell surface receptors for the secondary sites of

cancer metastasis. Soluble factor communication is often bidirectional between stroma and epithelium and

coordinated with other signaling molecules, such as extracellular matrices and integrins and other intracellular receptor signaling (e.g. steroid receptor). In some cases, additive or synergistic interactions could occur between various signaling cascades that could culminate to create a vicious cycle of positive feedback facilitating aggressive local cancer growth and metastatic spread to distant sites. The most commonly cited pathways involving soluble factors are summarized in Table 2. New insights into several of these pathways have provided exciting potential therapeutic targets.

Recruitment of neovascular endothelial cells to the proliferating cancer cells is thought to be a required for both the maintenance and stimulation of tumor growth. Thus, it is not surprising that the expression of vascular endothelial growth factor (VEGF) and their receptors is tightly regulated by androgen. The secretion of VEGF has been shown in both glandular and surrounding stromal cells. The resulting effect is stimulation of vasculogenesis by the action of VEGF on the endothelial component of the mesenchyme. Androgen deprivation has been shown to decrease VEGF expression by prostate cancer and is thought to be a mechanism of castration-mediated apoptosis. Additionally, finasteride, a 5 α-reductase inhibitor, has been shown to decrease VEGF expression as well as microvessel density in clinical BPH specimens. Direct inhibition of VEGF-mediated angiogenesis by thalidomide is another mechanism currently under clinical investigation affecting this pathway.

Dysregulation of the interleukin-6 (IL-6) pathway has been found in autoimmune disorders as well as different types of malignancy including multiple myeloma and prostate cancer. The complexities of the IL-6 signaling pathway were detailed in a recent review. High levels of IL-6 secretion from prostate fibroblasts and smooth muscle cells, as well as the tumor

cells themselves, is thought to be one mechanism of ligand-independent activation of the AR in prostate cancer cells. IL-6 mediated regulation of AR activation has been shown to occur via the PI3K-Akt, STAT3, and MAPK pathways and is proposed to be responsible for the androgen-independent progression of human prostate cancer. Interference with IL-6 signaling is a potential means of modulating the growth of advanced prostate cancer. Studies using an anti-IL-6 monoclonal antibody have shown tumoricidal effects in a murine model.

The insulin-like growth factor-I (IGF-I) pathway has been shown to be involved with malignant transformation in a variety of tissue types. Malignant prostate epithelial cells are sensitive to the surrounding IGF-I levels, regardless of their androgen-sensitivity status. Overexpression of IGF-I has been shown to drive neoplastic transformation of murine prostate epithelium, while antisense RNA to the IGF-IR inhibits prostate cancer proliferation and invasion. Other manipulations of the IGF axis with therapeutic potential include increasing IGF-binding protein expression, which has been shown to induce apoptosis in prostate cancer cells.

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, c-met protooncogene, were shown to be prevalently expressed by both localized and metastatic prostate cancer. Experimental evidence suggests that HGF/SF and c-met downstream signaling may regulate prostate cancer growth and metastasis through enhanced IL-6, androgen receptor, extracellular matrix and integrin interaction. PDGF and its receptor c-Kit are expressed in human prostate cancer and their interaction could also play a role in regulating prostate cancer growth. This opens up the opportunity of evaluating imatinib mesylate (STI571 or Gleevec), a tyrosine kinase inhibitor, which specifically blocks the cell surface PDGF receptor, c-Kit and its downstream signaling cascade. ErbB family members including EGFR, erbB2/neu HER2, erbB3 and erbB4

are known to have a role in prostate cancer progression through their interactions with a broad spectrum of soluble factors and their downstream converging signaling pathways. A large family of heparin bound growth factors, such as bFGF, VEGF, HGF/SF and HB-EGF could be targeted either individually or collectively through the inactivation of their co-receptor, perlecan (a heparan sulfate proteoglycan). Farach-Carson et al. (personal communication) provided experimental evidence by blocking perlecan with sequence-specific ribozyme, greatly diminishing the ability of prostate cancer to grow in the skeleton. The TGF-β family and their receptors play a pleiotropic role in prostate cancer. Since this growth factor family also affects EMT, angiogenesis, extracellular matrix turnover and host immune surveillance, a large body of literature describes the possibility of targeting TGF-β receptor pathways in altering prostate cancer behaviors.

Further understanding of the complexities of soluble factor-mediated stromal-epithelial communication may yield novel therapeutic targets for prostate cancer. Manipulation of these pathways in the native environment may prevent development or growth of primary malignancies while alterations in remote tissues may convert a previously fertile metastatic site into a hostile environment for tumor cells.

Solid extracellular matrices (ECMs) and integrin interactions promote cancer cell proliferation, survival and ability to adhere, migrate and metastasize

At the invasion front of tumor cell clusters, a noticeable derangement of ECM barriers often occurs (19, 30, 31). Interestingly, most of the key enzymes controlling ECM breakdown are not derived from tumor cells but rather from the host stroma, such as immune, inflammatory, endothelial and fibromuscular stromal cells. It is still unclear whether cancer cell invasion develops *before* or *after* interacting with host stroma, nor is it clear whether stroma response is subject to reciprocal regulation by cancer cells. Since maintenance of epithelial homeostasis requires the participation of stroma, it is likely that when epithelium changes the stroma inevitably follows (32). Cancer cells are likely to evolve continuously under the influence of products from deranged ECM barriers. Interactions between the soluble factors and/or the degraded and released solid tissue matrix proteins and cancer cells become possible due to the breakdown of tumor stroma barriers. ECM and its degradative products could signal cancer cells through their cell-surface integrin or non-integrin associated receptors and affect cell behaviors such as cell polarity, secretion, adhesion, motility and invasion and integrated cell functions such as proliferation, differentiation and survival. Integrins may have more complex roles by coordinating their actions with metalloproteinases and serine proteases, which together may increase cancer cell invasion, migration and extravasation into secondary sites of cancer growth (31, 32). The preferential usages of certain integrin isotypes (33), the "cross-talk" between soluble and solid matrix factors, cell contact in a 3-D structural scaffold and the downstream signaling pathways in prostate cancer cells during androgen-independent and bone metastatic progression could potentially reveal new therapeutic targets for cancer metastasis therapy. New therapeutic agents, either in the form of an antibody or a peptide, have been developed based on

their ability to interfere with ECM and cell-surface integrin interactions. Others were developed based on their interference with downstream converging signaling pathways originated from the soluble and solid tissue matrix-mediated signaling that determines cancer cell proliferation, survival and sensitivity toward drugs, hormones and/or radiation.

Transdifferentiation of prostate cancer cells to increased malignant potential by Epithelial to Mesenchymal Transition (EMT)

EMT is a fundamental process that determines body plane and polarity during embryonic development, where epithelial cells in the ectoderm migrate, undergo mesenchymal transition, invade and insert themselves between ectoderm and endoderm layers (16). EMT has been documented in breast, bladder and prostate cancers through increased expression of mesenchymal genes and is associated with increased cell motility, invasion and migration (34-36). We and others have observed increased expression of vimentin and N-cadherin but decreased expression of E-cadherin during prostate cancer progression to invasive phenotypes either through HER-2/*erbB2/neu* oncogenic transformation or through *in vivo* clonal interactions (37-39). A number of soluble factors, such as EGF/TGFα, TGFβ1, acidic FGF, and solid matrix such as collagen have been shown to induce EMT and associated phenotypic switches (16, 35, 40). The resulting cancer cells with mesenchymal phenotypes can secrete matrix metalloproteinases, become more responsive to the inductive growth factor and cytokine milieu in the surrounding tumor microenvironment, and acquire increased malignant potential by augmenting cell motility, migration and invasion. Figure 1 depicts stromal activation, EMT and its downstream epithelial activation, in response to the inductive cues from cancer microenvironment possibly mediated by activation of MEK, Src and PI3K activity, thus affecting

cancer cell proliferation, survival, motility and invasion (16, 41). Targeting EMT downstream pathways has been shown to reverse the morphologic and biochemical features of EMT in cancer cells, restore their differentiation and reduce cancer metastasis (42, 43).

Reactivation of stromal fibroblasts to myofibroblasts during cancer progression

Another morphologic and biochemical transition in cancer-associated stromal compartments, called "reactive" stroma, develops during disease progression (see Fig. 1). This process is shared by wound-healing and tumorigenesis (32, 44), where stromal fibroblasts are observed to undergo myofibroblastic transition both morphologically and biochemically. Although the origin of myofibroblasts remains controversial, it is heterogeneous and they may be recruited from host fibroblasts, vascular smooth muscle cells and pericytes (32). Rowley and colleagues (45) have provided evidence that "reactive" stroma respond to prostate cancer epithelium, resulting in elevated expressions of vimentin, smooth muscle α-actin, and calponin, characteristic of the myofibroblast phenotype, in the surrounding cancer-associated stroma. De Wever and Mareel (32) comprehensively reviewed the interplay between cancer and stroma and proposed that two tightly interactive pathways, called the efferent and the afferent pathways, ultimately determine the nature of the reciprocity between cancer and stroma.

In the *efferent pathway,* cancer cells trigger a reactive stromal response. Cancer cells could induce stromal response by the release or deposit of soluble factors such as TGFβ and PDGF in the cancer-associated microenvironment. In response to these factors, stromal fibroblasts undergo myofibroblast transition. This pathway appears necessary and could represent an early event in prostate and breast cancer progression. Evidence in support of this suggestion comes from the observation that *normal* stromal fibroblasts from fetal urogenital

sinus (i.e. embryonic prostate fibroblasts, (46)) and mammary gland (isolated from reduction mammoplasty, (47)), when co-inoculated with their respective prostate or breast tumor cells *in vivo, inhibited* their growth. By contrast, spontaneously immortalized prostate stromal cells (20) or cancer-associated (but not benign-associated) stromal cells (48) co-inoculated with prostate cancer cells *in vivo, induced* their growth, suggesting possible roles of stromal cells *after* activation or transdifferentiation. In these cases, normal stromal cells inhibited cancer growth through induction of cancer cell differentiation whereas transdifferentiated or altered stromal cells (the "reactive" stroma) promoted tumor growth and accelerated androgen-independent and bone metastatic progression. The growth stimulatory mode of stromal cells could result from the conversion or transdifferentiation of stromal fibroblasts to a reactive stromal population via the proposed efferent pathway.

Somewhat unclear in these models is whether the stromal reaction in response to tumor epithelial induction involves permanent genotypic changes. Intriguing experimental evidence indicates that permanent genetic changes occurred in tumor-associated stroma harvested from breast cancer specimens by laser captured microdissection (49). Mouse stromal cells outgrown from human prostate xenografts were reported to have consistent and identical chromosomal aberrations (50). These results, taken together, strongly support the possibility that *interaction with cancer cells can cause permanent genetic changes in cancer-associated stromal cells.* This suggestion is supported experimentally by our co-culture study where growing C4-2, an androgen-independent (but not androgen-dependent) human prostate cancer cell line, together with the human MG-63 osteosarcoma cell line under 3-D conditions produced consistent and stable chromosomal changes and phenotypic gene expression (27) (also see below).

The *afferent pathway* describes the cancer cell response to altered stromal cells in the cancer microenvironment. Several key effects of the myofibroblasts or "reactive" stroma on cancer cell behaviors have been suggested. Reactive stromal cells can exert a pro-invasive signal increasing the motility, invasion, and decreasing the apoptosis of cancer cells. They can mediate cancer pain through the release of cytokines or neuroendocrine factors. They can guide cancer cell perineural invasion and dissemination through the release of soluble and solid matrix factors (see review (32)). All of the above-described cancer phenotypes have been observed in human prostate cancer, underscoring the importance of this pathway in human prostate cancer progression. Exploring the intimate interactions between tumor and stroma and the dynamic transition of stromal cells during cancer progression could lead to more accurate targeting of this molecular process and new treatment protocols for prostate cancer metastasis.

The switch of osteomimicry and vasculogenic mimicry upon metastatic progression

Many of the features of cancer growth, development and dissemination are known to recapitulate embryogenesis. This is not surprising since cancer cells are pluripotent and have stem-cell-like properties capable of differentiating into and expressing phenotypes restricted to specialized cell types such as bone or endothelial cells. During cancer progression prostate or breast cancer cells can switch their gene expression profiles by mimicking bone (10) or endothelial (12) cells. This section reviews the biology and potential implications of osteomimicry and vasculogenic mimicry in cancer growth and metastasis.

Several proteins commonly associated with and or restricted to bone cells, such as osteocalcin (OC), bone sialoprotein (BSP), osteopontin (OPN), osteonectin (ON or SPARC),

osteoprotegerin (OPG), PTHrP, M-CSF, RANK and RANKL, were also found to be expressed by prostate cancer cells (11, 51-56). Moreover, a bone-homing metastatic human prostate cancer cell line of LNCaP lineage, C4-2B, was found to calcify *in vitro* under mineralizing conditions (56). The acquisition of new gene expression profiles by prostate cancer cells that mimic bone is called osteomimicry (10). Apparently this interaction is reciprocal. The gene expression profiles of normal cells surrounding the cancer epithelium at the primary (e.g. the reactive stroma) and the metastatic bone site were also found to undergo morphologic and biochemical changes (26, 57). Using the human osteosarcoma cell line MG-63 as a model, we demonstrated permanent genetic, morphologic and gene expression changes in MG-63 cells after exposure *in vitro* to a LNCaP-lineaged androgen-independent human prostate cancer cell line, C4-2, in co-culture under 3-D conditions (26, 57). These observations show the plasticity of both cancer cells and their surrounding stroma and the potential reciprocal inductive influences between them. More information is needed to determine the true inductive potential and plasticity of *normal* epithelium and stroma when exposed to cancer cells and the possible effect of *aging* and contribution by circulating stem cells on this cellular interaction process since prostate cancer is known to develop more frequently in the aging population and circulating stem cells have been found to reside in and play key roles affecting the growth and differentiation of many adult organs including bone and liver. Further understanding of epithelial-stromal interaction in the context of cancer development and progression will improve our capability to design therapies targeting both the tumor and tumor microenvironment interphase.

To understand the molecular basis of osteomimicry, our laboratory focused on investigating the mechanism of osteocalcin promoter switching during human prostate cancer

progression. These studies revealed that activation of transcription factors, Runx2, JunD/Fra-2 and Sp1, could play a role (51). It has been shown that a number of intracellular signaling pathways including cyclic AMP-responsive G protein coupling and vitamin D receptor mediated pathways could be involved in the activation of OC promoter activity (51, 58, 59). By exposing target cells to soluble growth factors such as bFGF, PTH and a rich bone matrix protein, collagen 1, OC promoter activation was observed. Since these soluble and matrix protein factors are deposited by cancer and their associated prostate or bone stromal cells in the microenvironment, we suggest that activation of intracellular signaling pathways and a coordinated switch of transcription factors may occur during prostate cancer progression, and is a likely molecular basis of osteomimicry in prostate cancer cells. The expression of bone-like proteins could confer the following advantages to prostate cancer cells: 1) Prostate cancer cells could behave like osteoblasts by participating directly in osteoclastogenesis where increased bone turnover is observed (10). This increased local bone turnover following prostate cancer bone colonization will create new sites to facilitate further growth and metastasis of prostate cancer cells. 2) Expression of OC and BSP by prostate cancer cells could enhance bone mineralization. These bone matrix proteins are capable of binding with high affinity to the mineral component of the bone, hydroxyapatite, and participate in the recruitment of osteoclasts and osteoblasts which affect bone resorption and deposition, respectively. 3) Osteomimicry may be required for prostate and breast cancer growth and survival in bone. Studies from prostate, breast and several other cancer types (60) indicate the ability of bone matrix proteins to confer increased cancer cell growth, adhesion, migration and invasion (54, 61, 62). Engagement of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ by the bone matrix protein SPARC resulted in enhanced VEGF production by prostate cancer cells (63). De et al. (63) proposed that VEGF and VEGFR2 interaction through an autocrine

loop stimulated prostate cancer cell growth and integrin activation to increase further prostate cancer cell adhesion and migration toward bone. Experimental tumor models inoculating an osteoblastic human prostate cancer cell line C4-2B (55) or an osteoblastic human breast cancer cell line ZR-75-1 (64) in immune-compromised mice revealed that targeting the interphase between cancer cells and bone using OPG or an endothelin A receptor antagonist, Astrasentan, respectively resulted in selective tumor shrinkage and reduced osteoblastic reaction in bone but did not affect tumor growth at other ectopic and orthotopic sites. These results suggest that "cross-talk" between cancer and bone specifically utilized the osteomimetic properties of prostate and breast cancer cells and that such communication is different from cancer interaction with its primary organ sites (e.g. the prostate and breast stroma environments). If so, then it is not surprising that a bone-directed targeting strategy would be highly selective and effective against *only* bone metastasis and that such a strategy would be ineffective against the growth of prostate cancer at the primary organ site or metastases to other visceral organs (see below). To further improve therapeutic targeting of prostate cancer local growth and distant metastasis, more comprehensive definition of organ-specific stromal microenvironments in the primary and visceral organs is urgently needed.

Vasculogenic mimicry describes the plasticity by which melanoma cells, when placed under ischemic conditions, form their own blood supply channels connecting with previously existing vasculature, express endothelial associated markers (vascular endothelial (VE) cadherin, CD34, endothelial cell-specific molecule) and form an extracellular matrix-rich vasculogenic network in 3-D culture (12). These properties were first described by Hendrix and colleagues (12) using a melanoma model. It appears that vasculogenic mimicry occurs in several other

cancer types including prostate cancer. The molecular mechanisms underlying vasculogenic mimicry by melanoma cells were investigated using microarray analyses and inhibitor studies. Results of these studies concluded that tyrosine kinase EPHA2, VE-cadherin, focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) are involved as integrated signaling pathways controlling vasculogenic mimicry (12). Since ischemia facilitates vasculogenic mimicry, it is likely that hypoxia, a condition commonly associated with tumor progression, may promote this morphologic and functional transition of cancer epithelial cells.

Anti-angiogenic therapy for cancer has not been highly effective despite the well-appreciated fact that tumor angiogenesis is a prerequisite for tumor growth. The origin of the endothelial network formed around tumor cells and its relative sensitivity to anti-angiogenic therapy is an important therapeutic concern. Realizing that one of the potential contributing cell types for tumor angiogenesis is the tumor itself, plus other cells not of obvious endothelial lineage, may provide a clue to the development of anti-angiogenic drugs targeting the heterogenous origin of the endothelial network in the tumor microenvironment. Vasculogenic mimicry in cancer needs to be further explored so more effective anti-angiogenic therapies can be developed.

3-Dimensional tissue organization regulates the polarity, secretion, gene expression and behavior of cancer cells

Cancer *in situ* does not grow as a 2-D adherent cell array, but as a 3-D closely packed organoid in close interaction with its microenvironment. A number of 3-D experimental models have been developed to assess how cell microenvironment regulates cell polarity, genetics and

behavior (17, 19, 65-67). Malignant breast cancer cells cultured on plastic yield the same phenotype and growth rate as non-malignant breast epithelium. When placed in a 3-D reconstituted extracellular matrix scaffold, however, malignant breast epithelial cells form an amorphous structure with unregulated proliferation, due in part to aberrant expression of integrins and epidermal growth factor receptor and the loss of ability to sense contextual cues from the surrounding microenvironment. Non-malignant breast epithelial cells, by comparison, will undergo growth arrest, form a polarized alveolar structure, and secrete milk (65, 68).

A 3-D assembly of cells can be conveniently constructed by growing prostate cancer and prostate or bone stromal cells under zero gravity simulated conditions (27, 67). In this model, our laboratory has shown the reciprocal interaction between prostate cancer and bone stromal cells whereby permanent and stable phenotypic and genotypic changes occur in prostate epithelial and bone stromal cells after cellular interaction under zero-gravity simulated conditions (27). For example, a non-tumorigenic human prostate cancer cell line, LNCaP, can be promoted to undergo androgen-independent progression and express a highly malignant potential, including the ability to invade locally and metastasize to the skeleton, only *after* cellular interaction with either prostate or bone stromal cells, or an extracellular matrix scaffold under 3-D growth conditions. This is remarkable considering that none of these changes were observed when co-culturing these cells under 2-D conditions. These findings recapitulate our previous observations *in vivo*, where we have observed *permanent* phenotypic and genotypic changes in prostate cancer epithelial cells, including androgen independence and bone metastasis, when co-inoculated and grown together with bone stromal cells as 3-D chimeric tumors (25, 69).

In addition to the breast and prostate cancer models described above, developing kidney, cartilage, heart, pancreas and ovary under 3-D conditions recapitulates the normal patterns of differentiation of these organs, suggesting again the parallelism between organ development and carcinogenesis. Both models require a committed cellular interaction under 3-D conditions. Although the precise mechanism of cellular interaction between cancer cells and soluble factor(s)/ECMs in the microenvironment remains speculative, most likely the underlying molecular basis controlling the progression of cancer resides in certain coordinated changes of gene transcription that occur in a highly temporal and spatially-regulated manner in response to inductive cues from the host microenvironment (19, 68). In a recent review, Ingber (19) elegantly summarized the principle of mechanochemical transduction of signals to control normal and malignant tissue differentiation, in which mechanical stretch of cells can trigger a host of coordinated chemical signals that could control cell growth, expansion of basement membranes, and orderly progression of morphogenesis and cytodifferentiation of the normal developing tissues. In carcinogenesis, a deregulated epithelial-mesenchymal interaction could cause accelerated turnover of basement membrane proteins and release of mechanical constraints on cancer epithelium so a deregulated growth, migration and differentiation program can ensue.

Prostate cancer skeletal metastasis: targeting tumor as well as its microenvironment

Men with advanced prostate cancer often undergo debilitating bone metastatic disease for which there is no effective therapy. To develop a sound approach tackling prostate cancer bone metastasis, many laboratories have begun evaluating the biology and molecular basis of prostate cancer bone metastasis. Using human prostate cancer cell lines and xenograft models, we can conclude that: 1) bone metastasis is conferred by specific cellular interaction between cancer and

host bone cells in which the growth and differentiation of prostate cancer and bone stromal cells can be reciprocally regulated (26, 57, 70) and certain chemoattractants produced by bone cells to recruit and retain prostate cancer cells in bone (71); 2) metastatic prostate cancer cells can express bone-like proteins (osteomimicry), participate in osteoclastogenesis and proliferate, invade and survive in bone microenvironment (10); 3) prostate cancer progression to androgen-independence and bone metastasis is accelerated by androgen withdrawal; 4) targeting bone and the interphase between prostate cancer and bone has yielded improved survival in mice with prostate caner xenografts (11) and human prostate cancer patients (72). Table 3 summarizes clinical experience with various therapeutic strategies co-targeting both cancer and its microenvironment with listed references.

Epidermal growth factor receptor (EGFR) emerged as an initial critical target. In an effort to repeat the success seen in other tumor types with gefitinib (Iressa), a small molecule that inhibits the kinase activity of the EGFR, a Phase II trial looked into the efficacy of single agent gefitinib in patients with advanced hormone refractory prostate cancer (HRPC) but failed to detect a significant response rate. The success of trial design was affected by patient selection and could be improved by obtaining additional data on the status of EGFR mutations since it has been reported that tumors contain mutated EGFR were more sensitive to growth inhibition by gefitinib.

Platelet derived growth factor receptor (PDGFR) has emerged as a dual target for epithelial cancer cells and bone stroma. PDGF and PDGFR are co-expressed in prostate cancer and neovasculature in metastatic sites. The binding of the ligand (PDGF) to its receptor

(PDGFR) results in inhibition of apoptosis but also accounts for the osteotropism of prostate cancer (10) . Imatinib mesylate (Gleevec) is a small molecule that binds to an ATP-binding site of the PDGFR kinase and blocks this particular signaling pathway. A Phase I trial of imatinib plus docetaxel found no single agent activity of imatinib but potential synergy for the combination. This concept is currently being addressed in a phase II randomized trial. Of note, preclinical data suggest that PDGFR-b kinase is a major mediator for increased tumor interstitial pressure (TIP), which hinders adequate delivery of cytotoxics to the targeted epithelial component. Imatinib given with paclitaxel effectively decreased TIP, which in turn led to increased uptake of paclitaxel and enhanced tumor cell kill.

Bisphosphonates are a class of drugs that target osteoclasts, and have been used with great success in disease states associated with increased osteolytic activity (osteoporosis, multiple myeloma). It is now appreciated that increased osteolysis accompanies metastatic prostate cancer, validating osteoclast may be a prime target. Preclinical data with the third generation bisphopshonate, zolendronic acid (Zometa), appear very promising in that regard. Currently, clinical interest in bisphosphonates has focused primarily on prevention/delay of skeletal related events (SRE) (fractures, need for therapeutic intervention, cord compression) and control of bone pain. A Phase III trial with zoledronic acid in patients with metastatic hormone refractory prostate cancer has found the drug to reduce the absolute risk of an SRE by 11% at a median follow up of 24 months. This has led to FDA approval for that particular indication. Of note, pamidronate has failed to provide equivalent results.

Atrasentan is a small molecule that blocks the receptor that mediates the effects of endothelin-1 (ET-1). ET-1 is a potent mitogen for osteoblasts and modulates nociception, thus

providing a target for the cancer-osteoblast interphase and control of bone pains. A prospective randomized, placebo-controlled Phase II trial investigated the efficacy of atrasentan at two different doses (2.5 mg and 10 mg po daily) in delaying time to progression (TTP) and providing adequate analgesia in patients with metastatic HRPC. Atrasentan at 10 mg vs. placebo was found to prolong median TTP (183 days vs 137 respectively, p=0.13) with very similar result for the 2.5 mg dose. Analysis of the data by actual treatment received (244/288 patients) found a statistically significant difference in median TTP (196 days vs.129 days, p=0.021) in favor of atrasentan. Median time to PSA progression was twice as long in the 10-mg atrasentan group compared with the placebo group (155 days *v* 71 days; $P = .002$). Quality of life measurements were not significantly improved in the treated arms. A Phase III trial with atrasentan has completed data accrual and results are awaited.

The idea of bone- and tumor-co-targeted therapy has been tested. A gene therapy trial focused on recombinant replication-defective adenovirus Ad-OC-TK (OC promoter-driven-herpes simplex virus-thymidine kinase co-expressed in both tumor and stromal cells) resulted in effective tumor lysis in both pre-clinical models and in patients in a Phase I trial. A Phase II trial in patients with hormone refractory prostate cancer with KAVE chemotherapy (ketoconazole/adriamycin alternating with vinblastine/estramustine) and then randomized patients who either responded or were clinically stable to continue with either adriamycin alone or adriamycin plus strontium^{-89}. The arm that received the chemotherapy/radiopharmaceutical combination had a median survival of 27.7 months vs. 16.8 months for those who received adriamycin alone (p=0.0014). These exciting findings await confirmation from a Phase III trial that is currently underway.

Angiogenesis has been proposed as a stromal target that can limit further progression of metastatic prostate cancer. A Phase II trial of bevacizumab, a monoclonal antibody that targets the vascular endothelial growth factor (VEGF), failed to produce significant clinical activity when used as single agent in patients with HRPC. Thalidomide has also shown anti-angiogenic effects (along with immunomodulatory activity). A Phase II trial of single-agent thalidomide found minor activity when used alone. Possible synergy with chemotherapy has also been proposed.

Finally, active immunotherapy may also target the stroma. GVAX, an allogeneic vaccine ex-vivo transduced to secrete GM-CSF, completed a phase II trial where it was noted that ICTP, a marker of osteolytic activity (typically elevated in patients with HRPC), remained stable or decreased in 70% of GVAX treated patients, thus raising the issue of additional yet unidentified targets in the bone stroma.

In conclusion, a number of targets that facilitate the cross-talk between stroma and prostate cancer cell have emerged. Combining therapeutic strategies that co-target two or more of these targets with or without concomitant cytotoxic strategies will usher in a new era of drug development in metastatic prostate cancer. The goal, quite an attainable one, will be to delay the symptomatic progressive metastatic phenotype and convert HRPC into a chronic disease where the "host" stroma cells and the "invading" prostate cancer cells have learned to co-exist in a balanced equilibrium.

Future Prospective

Cancer genotype and phenotype are influenced profoundly by microenvironment. Permanent genetic and behavioral modifications have been observed in both cancer and stromal cell compartments upon co-culture of these cells under 3-D conditions without the need of transferring foreign genes. These stably *induced* rather than *inherited* genetic and phenotypic changes, through reciprocal cellular interaction, can contribute to cancer progression. If the underlying assumption that cancer-microenvironment interaction dictates cancer progression is correct, this provides a molecular basis and immeasurable opportunities for therapeutic intervention to change the natural history of prostate cancer. Future pursuit of cancer-stroma interaction and altered stromal signature at the molecular level during disease progression will help improve our ability to diagnose, prognose and treat cancer. Ultimately, profiling the molecular signatures of cancer as well as its associated stromal components could provide new insights that will be applied and practiced in the future as the basis for personalized medicine.

§ Note in proof: Bhowmick et al. [Science 303: 848, 2004] have recently reported that by interrupting TGFβ signaling in prostate stroma modulates the oncogenic potential of adjacent epithelia.

Table 1 Human Prostate Cancer Cell and Xenograft Models to Study Androgen-Independent and Metastatic Progression of this Disease

Lineage-Related Progression Model	Commonly Used Cell Lines	Comments	References
LNCaP	C4, C4-2, C4-2B	Derived from LNCaP with increasing androgen-independent and bone metastatic potential	Thalmann, et al. Cancer Res 54: 2577, 1994; Prostate 44: 91, 2000
ARCaP	ARCaP sublines	Single cell cloned or selected after metastasis to bone	Zhau, et al. PNAS 93: 15152, 1996; Xu et al. Urology (Supp) 169: 81, 2003
PC-3	PC-3M,PC-3M-Pro4, PC-3M-LN4	Selected *in vivo* after orthotopic implantation of PC-3M cells	Stephenson, et al. JNCI 84: 951, 1992; Pettaway, et al. Clin. Cancer Res. 2: 1627, 1996
CWR22	$CWR_{22}R_{v1}$	Derived from hormone relapsed and CWR22 tumor grown in a castrated host	Wainstein,, et al. Cancer Res. 54: 6049, 1994; Sramkoski, et al. In Vitro 35: 403, 1999
RWPE-1	N-methyl-N-nitrourea (MNU)-treated sublines	Exposure of an immortalized human prostate epithelial cell line to MNU and developed sublines with increased tumorigenicity	Bello-DeOcampo, et al. Mut. Res. 480/481: 209, 2001
Xenograft Model	**Commonly Used Cell Lines**	**Comments**	**References**
MDA-PCa	MDA-PCa2a, MDA-PCa2b	Derived from bone metastatic specimens of a single patient	Navone, et al. Clin. Cancer Res. 3: 2493, 1997
LuCaP	$LuCaP_{35}$, $LuCaP_{35V}$, $LuCaP_{23\text{-}1}$, $LuCaP_{23\text{-}12}$	Developed from patients with prostate cancer metastasis to lymph node and liver	Ellis, et al. Clin. Cancer Res. 2: 1039, 1996; Corey, et al. Prostate 55: 239, 2003
LAPC	LAPC-8, LAPC-4, LAPC-9	Derived by implanting surgical specimens from patients into SCID mice	Klein, et al. Nat. Med. 3: 402, 1997
PC	PC-82, PC-133, PC-135, PC-295, PCEW	PC xenografts were established by growing primary prostate cancer or lymph node metastasis in nude mice	van Weerden, et al. Prostate 43: 263, 2000
DuCaP	DuCaP	A single cell line derived from the dura mater of a prostate cancer patient	Lee, et al. In Vivo 15: 157, 2001
VCaP	VCaP	A single cell line derived from the vertebra of prostate cancer metastasis	Korenchuk, et al. In Vivo 15: 163, 2001

Table 2 Most commonly cited soluble factor signaling pathways regulating prostate growth and differentiation

Soluble Growth Factor	Soluble Growth Factor Source	Growth Factor Receptor	Growth Factor Receptor Location	Function	Regulation Upon Androgen- independent Progression
VEGF	E, S	VEGFR-1 VEGFR-2	E, S	Angiogenetic factor	Correlate negatively with disease prognosis (van Moorselaar, et al
Mol. Cell Endo. 197: 239, 2002)					
bFGF (FGF-2)	S	FGF-2R	E, S	Angiogenetic factor	Correlate positively with disease prognosis (see ref. above)
HGF	S	c-met	E	Stimulates cell growth	Positively correlation with disease progression (Lail-Treker, et al. J.
Soc. Gyn. Invest. 5: 114, 1998; Kundsen and Edlund, Adv. Cancer Res. 2004 (in press).					
TGF-β	E	TGF-β I, II, III receptors	S	Induce apoptosis, increase angiogenesis, stimulate	Augmented expression upon androgen withdrawal (Blanchere et
al. J Steroid Biochem 82: 297, 2002) inhibit cell growth					
IGF-I	S	IGF-IR	E, S	Stimulates cell growth block apoptosis	Upregulation upon disease progression (Djavan, et al. World J
Urol. 19: 225, 2001; Prostate 58: 41, 2004) IL-6			E, S	IL-6R, sIL-6R	E, S Differentiation and
	Increasing IL-6 signaling during inhibition of apoptosis	Gp130		(soluble form) disease progression (Royuela, et al. J Path 202 : 41, 2004)	
KGF (FGF-7)	S	KGF-R	E	Stimulates cell growth	Stromal KGF expression responded to androgen (Planz, et al. J Urol
166: 678, 2001)					

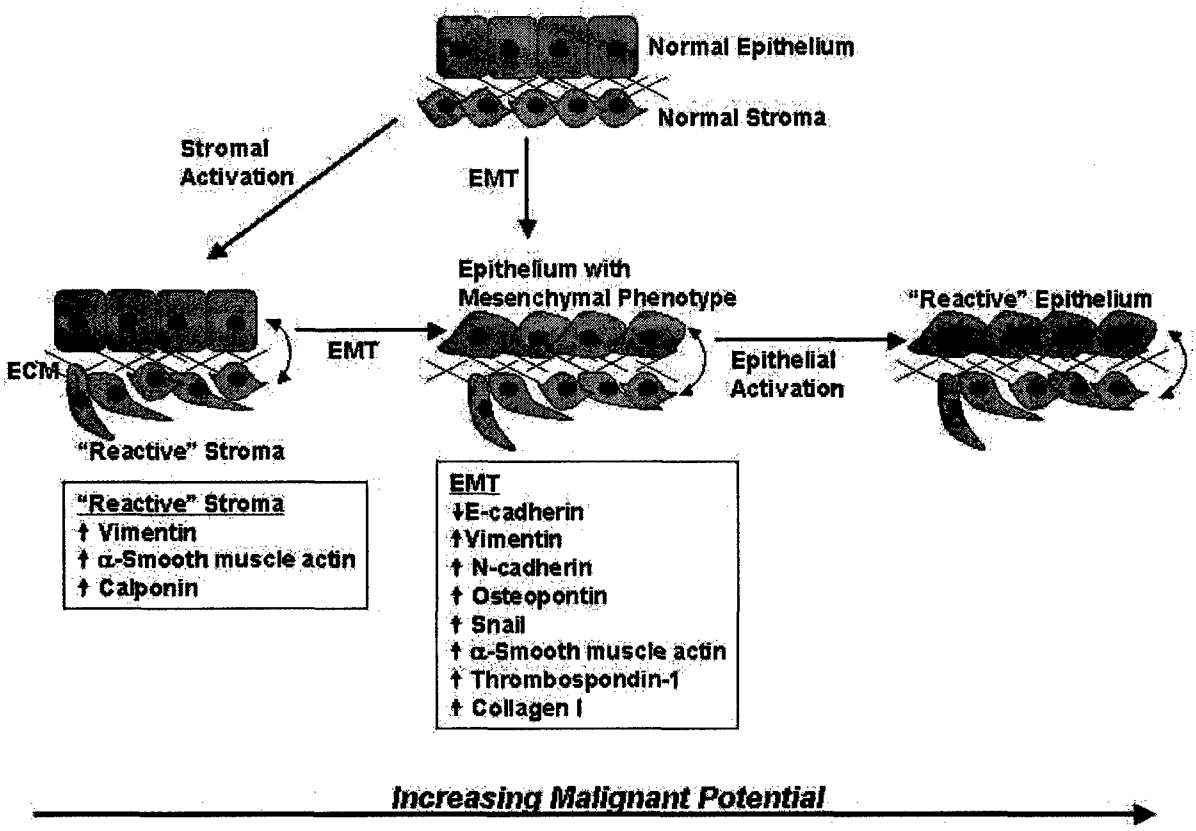
Table 3 Clinical experience of targeting cancer and its microenvironment

Drug	Target	Type of Trial	Comment	References
SOLUBLE FACTORS				
Gefitinib (Iressa)	EGFR	Phase II	minimal activity as single agent	Moore, et al. Ann. Oncol 5:326, 2002
Trastuzumab (Herceptin)	Her-2 neu	Phase II	trial ongoing, target of questionable clinical significance	
Imatinib mesylate (Gleevec)	PDFGR-b	Phase I	no activity as single agent, possible synergy with taxanes	Uehara, et al. JNCI 95: 458, 2003
BONE TARGETED				
Zolendronic acid (Zometa)	osteoclast	Phase III	11% ARR in SREs, FDA-approved for HRPC	
Atrasentan	osteoblast, PC	Phase II	trend towards delayed TTP	Nelson, et. al. Nature Med. 1:944, 1995 and Carducci, et al. J. Clin. Oncol. 21: 679, 2003
strontium-89	bone interface	Phase II	prolongs OS in chemoresponsive HRPC, when used a consolidation	Tu, et al. Lancet 357: 336, 2001
Gene therapy	osteoblast, PC	Phase I	concurrent co-targeting to both tumor and stroma using osteocalcin promoter	Kubo, et al. Human Gene Therapy 14: 227, 2003
ANGIOGENESIS				
Bevacizumab (Avastin)	VEGF	Phase II	minimal activity as single agent	Reese, et al. Proc. ASCO, 1999
Thalidomide (Thalomid)	bFGF, IL-8	Phase II	minimal activity as single agent, possible synergy with taxanes	Figg, et al. Clin. Cancer Res. 7: 1888, 2001; Semin Oncol 28: 62, 2001
IMMUNOTHERAPY				

GVAX	unknown	Phase II	antitumor effects coupled with decreased osteolytic activity, Simons, et al. 2003

Footnote: EGFR=epidermal growth factor receptor, PDGFR=platelet derived growth factor receptor, ARR=absolute risk reduction, SRE=skeletal related events, PC=prostate cancer cell, TTP=time to progression, OS=overall survival, HRPC=hormone refractory prostate cancer, VEGF=vascular endothelial growth factor, bFGF=basic fibroblast growth factor, IL-8=interleukin 8

Legend to Figure 1: Transition of normal prostate epithelial cells to increased malignant potential through cellular interaction with host stromal cells. There are potentially three different pathways a normal prostate epithelial cell can progress to become a cancer cell with uncontrolled growth and probability to disseminate. First, stromal activation to become "reactive" stroma which could be responsible for driving a pre-malignant prostate epithelial cell to undergo additional phenotypic and genotypic changes so that it acquires proliferative and survival advantages in the primary organ. Second, a pre-malignant epithelial cell, under the instruction from secreted soluble and insoluble matrix factors in the microenvironment, can undergo epithelial to mesenchymal transition (EMT), by expressing mesenchymal genes and gaining increased invasion, migration and metastatic potential; the epithelial cell often appears in the invasion front of the cancer, become amoeba-like, loss cell polarity and gain invasive and migratory properties in response to the chemical and haptotactic stimuli from the cancer immediate microenvironment. Third, additional genotypic and phenotypic changes may occur in prostate epithelial cells through an epithelial activation process. The "reactive" epithelium, in response to the continued inductive cancer microenvironment milieu either at the primary or at the secondary site of growth, could be the "lethal" clone of prostate cancer cells. Understanding cancer and microenvironment interaction at the cellular and molecular level could greatly help us in designing rational therapies for the treatment of human prostate cancer metastasis.


Normal Epithelium
Normal Stroma
Stromal Activation
EMT
Epithelium with Mesenchymal Phenotype
"Reactive" Epithelium
ECM
EMT
Epithelial Activation
"Reactive" Stroma
"Reactive" Stroma
↑ Vimentin
↑ α-Smooth muscle actin
↑ Calponin
EMT
↓E-cadherin
↑Vimentin
↑ N-cadherin
↑ Osteopontin
↑ Snail
↑ α-Smooth muscle actin
↑ Thrombospondin-1
↑ Collagen I
Increasing Malignant Potential

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